Novel Approaches to Treat Glioblastoma Multiforme

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INTRODUCTION

Glioblastoma multiforme (GBM), central nervous system (CNS) WHO Grade 4 astrocytoma, is an almost uniformly lethal disease with survival measured in months. Despite improved radiotherapeutic technologies and the use of the alkylating agent temozolomide, the majority of GBM patients succumb to their disease within approximately 17 months. This value has not been significantly altered in the past 20 years. Novel 'outside the box' therapeutic ideas will be required to prolong progression-free and overall survival in patients with an acceptable quality of life. This article discusses two recently completed clinical trials in primary diagnosed and recurrent GBM that were supported by the author's earlier pre-clinical studies.

THE APPROACH

GBM is a particularly lethal malignancy when compared to many other types of solid tumours. The primary treatment option for GBM, surgery followed by radiotherapy and temozolomide chemotherapy, was established 20 years ago by Stupp R et al.[1] Recurrent GBM is most often treated with the anti-angiogenic agent bevacizumab and a nitrogen mustard such as lomustine, which enhances 6-month progression-free survival.[2] None of these approaches are curative. Clearly, better treatment regimens, using previously untried novel cell biology concepts, need to be developed for patients in the primary diagnosis setting and in recurrent disease.

GBM is a heterogeneous disease, with multiple driving mutations present in different populations of cells simultaneously within the same tumour.[3-5] This largely defeats the modern 'personalised medicine' concept of one drug for one target. Common driving mutations in GBM include loss of the lipid phosphatase PTEN, expression of the truncated mutant activated epidermal growth factor receptor variant III (EGFR vIII), loss of p53, and overexpression of platelet-derived growth factor receptor A (PDGFRa).[6-8] More rarely, activating mutations in *B-RAF* are observed. Unlike other solid tumour types such as pancreatic and colon cancers,

GBM tumours do not express mutant RAS proteins. Thus, if an individual GBM tumour contains groups of cells, e.g., some expressing EGFR vIII and others expressing PDGFRa, any novel therapeutic approach will have to be developed that has a broad inhibitory spectrum against both primary driving oncogenes as well as any potential evolutionary escape/survival mechanisms.

An additional complication of treating GBM is the privileged environment within the CNS both restricting entry of therapeutic agents and by its unique cellular environment of neurons, astrocytes, microglia, and other cell types.[9–12] GBM, like all tumours, requires a supporting cast of different cell types to facilitate its growth, invasion, and therapeutic resistance. For GBM, one vital supporting player is CNS-localised macrophages, the microglia.[13,14] Minimally transformed astrocytes and microglia enter a symbiotic relationship where the transformed astrocyte releases growth factors, e.g., IL6, which activate the microglia. The activated microglia secrete additional growth factors and cytokines, which promote an inflammatory environment as well as the growth of the now well-established transformed astrocytes. As the transformed astrocytes progress through multiple cell cycles, genomic instability increases to the point where additional mutations/loss of tumour suppressors/gain of tumour promoters occur, and the transformed astrocyte eventually converts to become a malignant GBM tumour cell.[15]

Two novel therapeutic concepts have been developed by the authors' group, which attempted to address both the supporting role of microglia in GBM and interdiction of the multiple oncogenic drivers within any GBM tumour. Their initial concept was to suppress the actions of activated microglia, and for this they repurposed the multiple sclerosis drug dimethyl fumarate (DMF; NCT02337426).[16] Subsequently, to simultaneously attack GBM cells regardless of their oncogenic drivers, the group developed a combination of the liver cancer drug sorafenib, the anti-seizure medication sodium valproate, and the erectile dysfunction agent sildenafil (NCT01817751).[17]

DMF is approved for the treatment of multiple sclerosis. DMF breaks down in plasma to the active agent monomethyl fumarate (MMF).[18,19] The drug can inactivate T cells, but its mechanisms of action remain poorly understood. DMF has been shown to suppress the activities of microglia and astrocytes.[20,21] Microglia and astrocytes have important roles in the biology and progression of glial tumours, and repurposing DMF could be useful, changing glial cell viability and prolonging survival. For example, using microglia freshly isolated from glial tumours, they found that MMF significantly and rapidly reduced their production of IL-6, TNF-α, and TNF-β.[22,23] Treatment of microglia with MMF as a single agent for 24 hours killed glioma cells, and in tumours MMF, dramatically reduced the levels of microglia within the GBM tumour.

Based on the group's pre-clinical findings with DMF, they performed a Phase I trial to evaluate its safety and toxicity when combined with the standard Stupp protocol of concurrent radiotherapy and temozolomide followed by maintenance temozolomide.[16] Twelve patients were treated at three dose levels. No dose-limiting-toxicities were observed. The most common related adverse observations were haematologic (and typically seen with temozolomide alone): lymphopenia (58%), decreased CD4 count (17%), and thrombocytopenia (17%). The median progression-free survival (PFS) for all patients was 8.7 months, with no difference in PFS between those with stable disease (seven patients) or a partial response (four patients). The median overall survival (OS) was 13.8 months. For the six patients treated at the highest dose level, the median PFS was 11.8 months, and the median OS was 14.5 months. Due to the lack of sufficient power in the Phase I trial design, the group still does not definitively know whether DMF delivers any significant survival benefit to GBM patients.

The team then performed an additional series of pre-clinical studies in GBM cells where they combined DMF with another drug approved for MS, fingolimod.[23] Fingolimod is an analog of sphingosine-1-phosphate (S1P). Cells phosphorylate

fingolimod before it then, in an autocrine fashion, activates S1P receptors causing their proteolytic destruction. Immune cells lacking S1P receptors cannot migrate from lymph nodes to sites of myelin destruction.[23] MMF and fingolimod combined to kill, more than either drug alone, primary GBM cells and activated microglia, and their synthesis of cytokines. In mice treated with DMF and fingolimod for 14 days, no obvious normal tissue damage was noted. The drugs radiosensitised cells and enhanced the efficacy of temozolomide. Due to financial issues (i.e., the very high cost of purchasing dimethyl fumarate and fingolimod by the authors' institution) no translational studies in GBM patients have yet been proposed.

Sorafenib was originally developed to inhibit the proto-oncogenes *RAF-1* and *B-RAF* in the ERK1/2 MAP kinase pathway.[24,25] The kinase domain of the S/T kinase *RAF-1* has similarities to that of Y kinase SRC family proteins, and subsequently it was discovered that sorafenib inhibited Class III receptor tyrosine kinases such as PDGFRs and vascular endothelial growth factor receptors.[26] More recently the group demonstrated that sorafenib is a low affinity inhibitor of Hsp90 and Hsp70 family chaperones. Hence, the biological activities of sorafenib result in the drug having a complex mechanism of anti-tumour action.[27]

The authors' initial studies with sorafenib combined the drug with the epigenetic modulator family of drugs, histone deacetylase inhibitors (HDACi), in gastrointestinal tumour cells. This research initially resulted in clinical trials in liver cancer and in pancreatic cancer (NCT01075113; NCT02349867).[28] As would be expected with two agents that have a broad spectrum of action, the mechanisms by which tumour cells were killed are complex: death receptor signalling, ceramide generation, macroautophagy, reactive oxygen species generation, and calcium fluxes. Additional work demonstrated that sorafenib could interact with the HDACi and the anti-seizure medication sodium valproate to kill CNS tumour cells, including those derived from primary GBM and primary medulloblastoma. The mechanism of tumour cell killing was identical to that observed in gastrointestinal tumour cells. The sorafenib/HDACi combinations radiosensitised GBM cells, and the group demonstrated using molecular tools that at least a portion of sorafenib's anti-tumour activity was by inhibiting PDGFRa.[29-31]

One of the authors' earliest observations regarding the biology of sorafenib was that it caused an endoplasmic reticulum (ER) stress response.[31] Subsequently, they demonstrated that clinically relevant free concentrations of sorafenib inhibited the ATPase activity of the key chaperone regulating the ER stress response, GRP78 (BiP, HSPA5).[32] Under resting conditions GRP78 binds to PERK and IRE1, preventing them from signaling. When the levels of misfolded proteins in the ER increase, GRP78 dissociates from PERK and IRE1 to act as a chaperone, causing activation of PERK and IRE1. PERK, by the phosphorylation of serine 51, inactivates eIF2a resulting in the translation of approximately 90% of all mRNAs not being translated. Thus, proteins with particularly short half-lives, for example, the mitochondrial protective protein MCL1, have their expression reduced. For some genes, such as those for Beclin-1 and autophagy protein 5 (ATG5), eIF2a S51 phosphorylation increases their translation. Enhanced levels of Beclin1 and ATG5 would act to facilitate macroautophagy and the degradation of misfolded proteins. Once the misfolded proteins are degraded via autophagy, GRP78 re-associates with PERK, shutting of the ER stress response, and eIF2a is dephosphorylated by PP1.

Sildenafil is a phosphodiesterase 5 (PDE5) inhibitor.[33] It is used both as a medication for blood pressure and for erectile dysfunction. Inhibition of PDE5 results in elevated levels of its substrate cyclic GMP and activation of protein kinase G (PKG).[34] The biological actions of PKG are pleiotropic. PKG can act to increase expression of nitric oxide synthase, whose production of NO causes relaxation of smooth muscle in the cardiovascular system.[35] In tumour cells, which generally produce orders of magnitude greater levels of reactive oxygen species than non-transformed cells, NO can

interact with oxygen radicals and hydrogen peroxide to generate peroxynitrite ($ONOO^-$), a short-lived but lethal free radical.[36] Peroxynitrite causes lipid peroxidation, protein oxidation, and protein nitration, as well as DNA damage. Sildenafil synergises with sorafenib to kill tumour cells *in vitro* and *in vivo*, and this requires PKG signalling and the production of NO. This work resulted in a Phase I trial combining regorafenib and sildenafil in solid tumour patients (NCT02466802).[37] Sildenafil has also been recognised as an inhibitor of plasma membrane drug-efflux pumps responsible for the blood–brain barrier and chemotherapy resistance, e.g., ABCB1 and ABCG2.[38,39] Hence, the three-drug combination of sorafenib, valproate, and sildenafil has multiple overlapping two-drug combination synergies that collectively will act to kill tumour cells.

In the Phase II trial 'Sorafenib, Valproic Acid, and Sildenafil in Treating Patients with Recurrent High-Grade Glioma' (NCT01817751), the authors' combined sorafenib, valproate, and sildenafil based on validated concepts from their pre-clinical data, and that sildenafil may prevent the efflux of drugs out of the GBM cells.[17] At the end of the trial, 33 patients were available for evaluation. The most frequently observed negative sequela, as *a priori* expected, was skin rash. A statistical difference in OS was seen between patients with ECOG PS of 1 versus 2. Based on the authors' pre-clinical studies, the trial also examined the expression of PDGFRa and GRP78 in each patient's tumour as both are targets of sorafenib. OS was not different between tumours expressing high levels of PDGFRa compared to those with low levels ($p<0.07$). However, for the chaperone GRP78, OS was significantly higher for patients expressing low levels of the chaperone ($p<0.0026$). Tumours expressing high levels of GRP78 were associated with a shorter survival time than those expressing low levels of GRP78.

Of perhaps greater interest to the wider field, with respect to the authors' findings, is the extended survival of five patients (~15%) in the group who had the lowest protein levels of GRP78.[17] For all patients the median PFS was 3.7 months and OS 10.0 months. For the surviving five patients in the Kaplan-Meier tail, the mean value for PFS was 24.9 months (~2 years) and the mean OS value was 73.6 months (~6.1 years). The authors do not have a complete understanding as to how and why these five patients have extended survival, other than that they all had the lowest GRP78 levels. The five patients with lower GRP78 levels (15.2%) presently have a mean PFS of over 2-years and mean OS of over 6-years, and they all remain alive. This is three-times as many long-term survivors as the team would have predicted. The long-term survivors were four European-heritage males and one African American, both representing 16.7% of their respective populations within the trial.

GRP78 is predominantly found in the ER and PM, where it is known to act as a chaperone and as a regulatory protein required to maintain signalling through multiple intracellular signal transduction proteins, and to a lesser extent GRP78 is in the nucleus, where it acts as a co-transcription factor.[40] At present the authors do not know which sub-population of this chaperone plays the most important role in mediating resistance to sorafenib plus valproate plus sildenafil, though they postulate it is likely to be the GRP78 populations both in the ER and in the PM. High levels of GRP78 are significantly associated with lower PFS and OS, but it is not known whether other chaperones in the HSP70 family, like GRP78, or those in the HSP90 family, also play a role in modulating ER stress signalling by PERK/PKR-eIF2a and are additional significant correlates to be examined in future studies.

The essential autophagosome-regulatory protein ATG16L1 has two isoforms. Although adult glioma survival rates for people of European and African heritage are similar, the team know that Europeans trend to express the ATG16L1 A300 isoform and those of African heritage trend to express the ATG16L1 T300 isoform.[41] People homozygous for the T300 isoform are more able to facilitate autophagosome formation and digest antigenic materials in the gastrointestinal tract, which is a reason

why African Americans are less frequently diagnosed with Crohn's disease.[41,42] Hence, as a future long-term goal, the authors will need to statistically define whether the expression of GRP78 correlates with the isoform status of ATG16L1 to influence PFS and OS. This may provide additional clues for future studies as part of grant submissions in adult glioma.

CONCLUSION

The team at Virginia Commonwealth University has been very fortunate to not only translate into the clinic two investigator-initiated trials in GBM, but also perform many other trials in a variety of solid tumour types. Cancer developmental therapeutics by individual investigative teams, scientists, and physicians often encounter Himalayan sized obstacles preventing the team from taking any concept from the bedside to the bench and back to the bedside. First, there are 'technical issues' of obtaining drugs from drug companies who may not wish to collaborate with each other or further their development of a particular drug, with compromises therefore having to be made by investigators to use generic drugs, e.g., sodium valproate, over more expensive proprietary drugs in the same class such as vorinostat. Second, receiving regulatory approval from the FDA and local IRB can delay translation, particularly if the drugs have overlapping normal tissue toxicities that will require careful lead-in dose escalation approaches in the clinical protocol.

Third, with the first two issues paling into insignificance, is that translating a concept from the bench to the bedside must consider the cost of patient care and having all the necessary protocols overseen by regulatory committees within the academic cancer center. For example, a small 3×3 two-agent Phase I trial, with *gratis* supply of drugs, still has regulatory and healthcare costs that run into the many hundreds of thousands of dollars. The cost of a two-drug Phase I trial where both drugs must be commercially purchased can run into many millions of dollars, which is prohibitively expensive for any academic cancer centre. Hence, in an ideal world for GBM patients, who experience rapid morbidity and mortality, conceptually, scientists and physicians need to 'think outside the box' to increase the number of rapidly deployable therapeutic options. Perhaps, in the future, novel drug combinations that are known to have benign toxicity profiles and have exhibited broad anti-cancer effects in other solid tumour types could be more rapidly processed through the standard approvals process, including new laws that facilitate the billing of trial drug and healthcare costs to insurance, resulting in the more rapid delivery of new GBM therapeutic options.

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