Glioblastoma quo vadis: Will migration and invasiveness reemerge as therapeutic targets?

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Abstract: PURPOSE The purpose of the current review is to highlight, on one hand, the fact that the migratory pattern of glioma cells is the major obstacle to combat them with chemotherapy, and on the other one, the new treatment strategies to overcome this obstacle. METHODS This review surveys several membrane and extracellular molecules involved in glioma cell migration, invasiveness and resistance to apoptosis. RESULTS This review focuses on signaling pathways implicated in the positive regulation of glioblastoma cell migration, including glutamate and ion channel networks, microtubes and membrane-derived extracellular vesicles (EV) containing microRNAs. Glioma cells release glutamate to the extracellular matrix, inducing neuronal cell death, which may facilitate glioma growth and invasion. Glioma cell migration and invasion are further facilitated through ion channels and transporters that modify cellular volume. Microtubes and EV promote connections and communication among glioma cells and with the microenvironment and are associated with progression and resistance to therapy. Potential therapies linked to these pathways for glioblastoma are being developed. CONCLUSION Our view is evolving from an intracellular view of the complex intracellular signaling pathways to one of orchestral machinery, including connections between heterogeneous tumoral and nontumoral cells and with the microenvironment through channels, microtubes, and extracellular miRNA, generating different signals at different times. All of these elements give rise to a new perspective for the treatment of glioblastoma.

Glioblastoma *quo vadis*: will migration and invasiveness reemerge as therapeutic targets?

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Running Title: Migration and invasiveness in glioblastoma

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ABSTRACT

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Methods

This review surveys several membrane and extracellular molecules involved in glioma cell migration, invasiveness and resistance to apoptosis.

Results

This review focuses on signaling pathways implicated in the positive regulation of glioblastoma cell migration, including glutamate and ion channel networks, microtubes and membrane-derived extracellular vesicles (EV) containing microRNAs. Glioma cells release glutamate to the extracellular matrix, inducing neuronal cell death, which may facilitate glioma growth and invasion. Glioma cell migration and invasion are further facilitated through ion channels and transporters that modify cellular volume. Microtubes and EV promote connections and communication among glioma cells and with the microenvironment and are associated with progression and resistance to therapy. Potential therapies linked to these pathways for glioblastoma are being developed.

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Our view is evolving from an intracellular view of the complex intracellular signaling pathways to one of orchestral machinery, including connections between heterogeneous tumoral and nontumoral cells and with the microenvironment through channels, microtubes, and extracellular miRNA, generating different signals at different times. All of these elements give rise to a new perspective for the treatment of glioblastoma.

Introduction

Gliomas, including the most common variant, glioblastoma, are the most common primary malignant brain tumors, with an annual incidence of 5.26 cases per 100,000 population or 17,000 new diagnoses per year [1]. Glioblastoma remains one of the most lethal forms of cancer. The high spatial and temporal glioblastoma heterogeneity due to functionally diverse cell types, chaotic leaky vasculature and tumor cell infiltration contributes to poor prognosis in patients [2]. The current understanding of the biology and genetics of glioblastoma has greatly improved in the past two decades. Two major multi-institutional programs, the Glioma Molecular Diagnostic Initiative and The Cancer Genome Atlas (TCGA), have pursued comprehensive genomic characterization of a large number of clinical glioma samples using a variety of technologies to measure gene expression, chromosomal copy number alterations, somatic and germline mutations, DNA methylation, microRNA, and proteomic changes. These data will facilitate the discovery of therapeutic and diagnostic targets and pathway candidates, validation of research and clinical observations and generation of unanticipated hypotheses that can advance the molecular understanding of this lethal cancer [3]. Sturm et al. identified six epigenetic glioblastoma subgroups displaying characteristic global DNA methylation patterns, harboring distinct hotspot mutations, DNA copy-number alterations, and transcriptomic patterns [4]. Brenann et al. provided a comprehensive and in-depth overview of the genetic, epigenetic and transcriptional profiles associated with glioblastoma [5]. The new WHO 2016 classification, therefore, is not only based on the histological features of the tumor but also includes isocitrate dehydrogenase (IDH) mutation and chromosomal arms 1p and 19q codeletion status to classify diffuse gliomas of adulthood [6]. However, each individual glioblastoma should be considered as an ensemble of molecularly distinct subclones that reflect a spectrum of dynamic cell states. It is becoming clear that

glioblastoma is a composite of multiple cell populations in the same tumor, and this heterogeneity

makes the design of effective treatment modalities challenging [7,8].

Diffuse glioma genesis

In the context of gliomagenesis, glial cells were once considered the only dividing cells in the

postnatal brain, making these cells the only brain cells susceptible to transformation [9,10]. It is

now known that both multipotent and self-renewing neural stem cells and self-renewing glial

progenitor cells produce astrocytes and oligodendrocytes in limited regions of the adult brain

including the subventricular zone, the subgranular zone of the dentate gyrus and possibly other

minor stem-cell niches throughout the brain [11-13]. These stem cell and progenitor elements, in

addition to differentiating into adult glia, are now thought to constitute a substrate for neoplastic

transformation, termed glioma initiating cells or glioma stem cells (GSCs), a small population of

self-renewal, therapy-resistant and slow-dividing malignant cells inside the main tumor bulk.

Calabrese et al. found that a significant proportion of vessel-associated Nestin+ tumor cells are

proliferating and that these cells are distributed throughout tumors, from all regions of the

cerebrum and cerebellum [14]. In the early 70s, Pierce proposed that tumors arise from

carcinogenic events occurring in stem cells of a particular tissue that cause a defect in the control

of normal stem cell function, including self-renewal and differentiation [15]. In the adult brain,

neural stem cells commonly possess features associated with gliomas, including a robust

proliferative potential, high motility, diverse progeny, associations with blood vessels and white-

matter tracts and an immature gene expression profile characterized by expression of nestin,

epidermal growth factor receptor (EGFR), Notch and PTEN, telomerase activity, and Hedgehog

and Wnt pathway activities [16-19].

GSCs expresse nestin (an intermediate filament protein) and CD133 (also known as prominin 1, a

transmembrane glycoprotein), factors associated with neural stem cells, although some GSCs are CD133-negative [20]. Although CD133 expression seems to be related to stemness, it might only indicate an intermediate, adaptive state of a cell rather than a stable phenotype [21]. A tumor is therefore a caricature of normal tissue and appears to be undifferentiated because of the preponderance of undifferentiated proliferating stem cells in relationship to the number of cells that have differentiated and are post-mitotic.

Stem and progenitor cells in the central nervous system may be particularly at risk for malignant transformation since they constitutively activate the cellular machinery necessary for tumor initiation, progression or both (including growth factor receptor EGFR, PI3K, Akt, mTOR, Ras, mitogen-activated protein kinase (MAPK) members). Thus, neural stem cells may represent the path of least resistance in tumorigenesis since these cells already have the ability to bypass apoptosis and senescence. As a result, neural stem cells may require less than the estimated four to seven mutations needed to induce malignant change in a differentiated cell [22]. In gliomas, as in several solid tumors outside the central nervous system, only a small fraction of the total population of cancer cells are clonogenic cancerous neural stem cells. However, this resistant population is considered the primary cause of glioblastoma invasion and responsible for the maintenance of a minimal residual disease, which commonly leads to post-therapeutic recurrence [23]. Evidence from human gliomas and rodent glioma models indicate that the glioma vascular niche supports glioma stem cell maintenance and nurtures tumor growth by offering nutrients necessary for unlimited glioma stem cell proliferation [14,24]. Several embryonic signaling pathways, such as Notch, Hedgehog and Wnt/b-catenin, have been reported to help maintain the GSC pool and thus provide potentials target for treating glioblastoma [25]. Paw et al. [26] and Lee et al. [27] recently provided an overview of the specific signaling pathways that regulate glioblastoma stem cell maintenance and migration.

On the basis of their histological appearance, gliomas have been traditionally classified as astrocytic, oligodendroglial or ependymal tumors and assigned WHO grades I-IV, which indicate different degrees of malignancy. Diffusely infiltrating gliomas in adults are now separated into three overarching tumor groups with distinct natural histories, responses to treatment and outcomes: IDH-mutant, 1p/19q co-deleted tumors with mostly oligodendroglial morphology that are associated with the best prognosis; IDH-mutant, 1p/19q non-co-deleted tumors with mostly astrocytic histology that are associated with intermediate outcome; and IDH wild-type, mostly higher WHO grade (III or IV) tumors that are associated with poor prognosis [28].

Treatment

The current standard treatment for glioblastoma includes surgical resection, followed by radiotherapy plus concomitant temozolomide (TMZ) followed by six cycles of maintenance TMZ [29,30]. These treatments allow a median overall survival in the range of 16 months in clinical trial patient populations, but are never curative. Indeed, less than 10% of the 77,000 patients diagnosed each year with glioblastoma in the USA and Europe will survive more than 5 years. This dismal prognosis reflects the high rate of tumor recurrence approaching 100% which is linked to the profoundly infiltrative nature of this tumor, rendering curative surgical resection elusive, and its relative resistance not only to the conventional treatments of genotoxic radiotherapy and chemotherapy, but also more recently to targeted therapies. Antiangiogenic therapies have dominated the clinical trial landscape of glioblastoma. However, it has now become clear that this approach is hopeless, at least with a view to prolonging overall survival. Indeed, while anti-angiogenic treatment targeting VEGF exerts anti-edema effects in glioblastoma which may translate into clinical benefit in subgroups of patients, no clinical trial has demonstrated a survival benefit afforded by anti-angiogenic agents.

Glioblastoma migration: What is new?

In a previous review we highlighted the complex dynamic processes governing glioma cell migration in general and in glioblastoma in particular [31]. This dynamic process includes, among others, i) cell adhesion to numerous components of a modified (when compared to normal parenchyma) extracellular matrix (ECM); ii) cell motility, which involves the reorganization of the actin cytoskeleton, primarily through modifications in the field of integrin network–ECM component interactions; and iii) invasion, which involves the degradation of matrix proteins by tumor-secreted proteolytic enzymes, primarily serine proteases, cathepsins and metalloproteinases (MMPs). Admittedly, no effective treatments for human glioma patients have evolved from these avenues of research.

In the present review, we analyzed the recent literature on new promising therapies to combat glioblastoma with a focus on two pathognomonic features of glioblastoma biology, i.e., glioblastoma cell migration and invasion through the brain parenchyma and, associated with that, the apparent intrinsic resistance of migrating glioblastoma cells to pro-apoptotic stimuli, including cytotoxic chemotherapy and radiotherapy.

We focused in the current review on glutamate, ion channels, tumor microtubules and several signaling pathways. While there is a myriad of signaling pathways that influence the migratory behavior of malignant astrocytes [31], we decided to restrain ourselves on the "glutamate pathway" because we are convinced that impairing this malignant astrocyte-migration avenue of invasion within the brain parenchyma would actually contribute therapeutic benefits to the patients. We detail below why we believe in this avenue to combat malignant gliomas.

Interaction of glutamate, glutamate transporters and glutamate receptors

The release of the neurotransmitter glutamate from glioma cells has been well studied *in vitro* and *in vivo* as well as in glioblastoma patient populations [32-34]. The Na^+-independent, Cl^--dependent, cystine/glutamate exchanger system (SXC) is the only transporter identified in glioma cells that has the ability to release large amounts of glutamate [32]. Glutamate released from malignant glioma cells into the extracellular space has been linked to seizure induction, and at higher concentration, glutamate is thought to induce neuronal cell death, mediated mainly by prolonged activation of N-methyl-D-aspartate (NMDA) receptors, but also via α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors resulting in Ca^{2+} influx and Ca^{2+}-mediated cell death (Figure 1) [35-37]. In the healthy brain, excitatory amino acid transporters (EAAT), located on astrocytes (EAAT1 and-2) and neurons (EAAT2), are responsible for the majority of glutamate uptake, and astrocytes can rapidly reduce toxic concentrations of extracellular glutamate. Glioma cells lack EAAT-mediated glutamate transport which contributes to the elevated peritumoral levels observed in glioma patients and in animal models of glioma [38]. Moreover, Robert and Sontheimer hypothesized that glioma cells alter peritumoral astrocyte EAAT function, preventing the re-establishment of glutamate homeostasis, as typically occurs in the healthy brain [38]. Investigations effectively discovered that glioma cells lack EAAT2-mediated glutamate transport [39]. These findings were corroborated by microarray analyses of human glioma biopsies demonstrating the absence of EAAT2 expression in patient glioma tissue [39].

By contrast, glioma cells express NMDA, a-amino-3-hydroxy-5-methyl-4-isoxaole propionic acid (AMPA) and metabotropic (mGlu) receptors [40]. However, van Vuurden et al. showed that on protein level, AMPA-type glutamate receptor subunits are variably expressed in glioblastoma and

are overall down-regulated as compared to the normal brain tissue [41]. These authors further reported that down-regulation of AMPA receptor expression and mislocalization of these receptors enables the cells to survive in a high glutamate environment without undergoing excitotoxic cell death themselves [41].

Glutamate stimulates the growth, migration and invasion of neuroglial tumor cells through activation of Ca^{2+}-permeable glutamate AMPA receptors on glioma cells in a paracrine and autocrine manner (Figure 1). AMPA receptor expression correlates with an increase in surface expression of β1 integrin, a molecule implicated in glioma invasion, and AMPA receptor stimulation increases glioma cell de-adhesion from surrounding cells. Glial AMPA receptors activate focal adhesion kinase (FAK) which influences cytoskeletal rearrangement and focal adhesion contact formation [42].

Additionally, in response to reactive oxygen species (ROS), glioma cells upregulate SXC activity to provide intracellular cysteine for enhanced glutathione (GSH) synthesis, an important intracellular antioxidant that promotes cell survival [43]. Increased GSH neutralizes ROS, preventing cell destruction triggered by hypoxia; inhibits apoptosis signaling, protecting cells from degradation; and via ROS antagonism, may play an important role in the development of resistance to radiotherapy and chemotherapy [44].

Roles of ion channels, transporters and exchangers

Increasing evidence suggests that cellular migration and invasion in cancer cells in general and glioma cells in particular results from shape and volume changes facilitated by ion channels and transporters [45-48]. Glioma cells principally use voltage-gated chloride and potassium channels as well as the KCa family of Ca^{2+}-activated potassium channels ([47] for a review of this topic)

(Figure 2).

Potassium channels

Among ion transporters, potassium channels show the highest variability and the most frequently altered pattern of expression in many tumor types [49]. Cell cycle, proliferation, migration, invasion, angiogenesis and apoptosis are all processes that can be modified by the expression of potassium channels [50]. Potassium channels have been implicated in every step of adhesion and migration [51]. Interactions of potassium channels with crucial contributors to cell adhesion, such as FAK and cortactin, an actin-interacting protein implicated in the cytoskeletal architecture and often amplified in several types of cancer, have been reported (Figure 2) [52]. Potassium channels also associate with the main contributors to cell adhesion signaling, integrins. The most important understood mechanisms by which particular potassium channels contribute to cancer initiation and progression have recently been reviewed [48]. Here we focus on the literature on potassium channels involved in glioma progression and migration, e.g. Kv1.3 and Kv1.5 [53-55], Kv10.1 [56-58], Kv11.1 [59], KCa1.1 [60,61] and KCa3.1 [62] (Figure 2).

The Kv1.3, Kv10.1, and Kv11.1 channels are the best studied voltage-gated K channels in the context of cell migration. They are of particular importance for cells of the immune system and tumor cells, including glioblastoma cells, constituting potential diagnostic or prognostic markers or even therapeutic targets [48,50].

Kv10.1 (also known as EAG1, ether-à-go-go and KCNH1), a voltage-gated potassium channel that is virtually absent from normal cells outside the central nervous system, is expressed in limited areas of the brain (hypothalamus, hippocampus, cerebral cortex, cerebellum and olfactory nerve) but highly expressed in different cancers, including brain metastases and gliomas [58]. Bai et al.

also observed Kv10.1 overexpression in both glioma cell lines and clinical glioma samples [56]. In

several different cancers, Kv10.1 enhances proliferation. It has also been shown to be required for

the maintenance of growth associated with the p53/miRNA34/E2F1 regulatory pathway in

SHSY5Y human dopaminergic neuroblastoma cells [63]; this channel up-regulates HIF-1 and

VEGF in the tumor environment [64] and increases migration [65]. Kv10.1 expression in Chinese

Hamster Ovary (CHO) cells affects cytoskeletal organization, which might influence cancer cell

proliferation, migration and metastasis [65].

A correlation between dismal prognosis and Kv10.1 expression in patients with brain metastasis

from different carcinomas has been observed, and metastatic cancer patients receiving tricyclic

antidepressants (such as imipramine, chlorimipramine, citalopram and amitriptyline) showed a

significantly longer median overall survival than patients not treated with these agents [58].

Kv11.1 (also known as HERG or KCNH2) was among the first voltage-gated channels directly

associated with cancer [66]. Further, Kv11.1 blockers impair the proliferation of tumor cells [67].

Kv11.1 physically interacts with β1 integrin at the plasma membrane of human cancer cells to

regulate survival and migration [68] (Figure 2), and this channel has also been implicated in the

regulation of apoptosis of human glioblastoma cells (see below) [69].

The Kv1.3 and Kv1.5 channels modulate Ca^{2+} signaling and cell volume and have been analyzed in

a number of cancer cell types [70]. The levels of expression of the Kv1.5 and Kv1.3 channel

subtypes vary between different glioma groups, and differential expression of Kv1.5 according to

malignancy grade has been noted [53]. Kv1.5 also contributes to the resistance to pro-apoptotic

stimuli of cancer cells, including gliomas (see below).

The calcium-activated potassium channels KCa1.1 and KCa3.1 are important for the migration of

glioma and glioma stem cells [62,71,72]. The REepository of Molecular BRAin Neoplasia DaTa

(REMBRANDT) database allowed to determine that the gene KCNN4, which encodes KCa3.1, is

overexpressed in 32% of gliomas, and that its expression correlated with short survival [71]. Turner

et al. generated U251 glioma cells that stably express an inducible knockdown shRNA to

experimentally eliminate KCa3.1 expression [71]. Subjecting these cells to a combination of *in*

vitro and *in situ* invasion assays revealed that KCa3.1 expression may enhance glioma invasion and

either specific pharmacological inhibition with TRAM-34 or elimination of this channel by gene

silencing impairs invasion. Importantly, after intracranial implantation into SCID mice, ablation of

KCa3.1 with inducible shRNA from U251 cells resulted in a significant reduction in tumor

invasion into the surrounding brain [71].

Migration of glioblastoma cells can be stimulated by ionizing radiation (IR) [73]. Steinle et al.

accordingly analyzed the IR-induced increase in Ca^{2+}-activated potassium channel activity and its

effect on the migration of T98G and U87MG cells [74]. IR dose-dependently stimulated migration

of glioblastoma cells, which in turn was sensitive to the channel inhibitor paxilline. These authors

concluded that IR stimulates potassium channel activity, resulting in the activation of

Ca^{2+}/calmodulin-dependent protein kinase II and enhanced glioblastoma cell migration [74].

Chloride channels

Approximately 20 years ago, Sontheimer proposed that the invasive migration of human glioma

cells into the normal brain parenchyma requires shape and/or volume changes [75]. Chloride

currents may contribute to such shape-volume changes by affecting net salt fluxes across glioma

cell membranes, with accompanying water efflux, resulting in cell shrinkage that is conducive to

glioma cell migration through minute extracellular spaces of the brain [76]. Sontheimer and

collaborators subsequently performed a direct examination of Cl⁻ dynamics in human glioma cells

and its functional relationship to cell biology [77]. First, these authors observed that invasive tumor

cells become morphologically polarized and develop membrane protrusions (Figure 2). During this process, invasive glioma cells alter their cell shape and volume to fit into the narrow extracellular spaces available and invade the brain parenchyma. These features include secretion of Cl^- and K^+ through ion channels localized to lipid raft domains on invadopodia and water passively flowing through water channels or aquaporins [46]. In addition, the interplay between Na^+-K^+-Cl^- cotransporters and Na^+/K^+ ATPase leads to active accumulation of K^+ and Cl^-, establishing a gradient for KCl efflux (Figure 2) [46].

Here, we do not review the roles of other channels involved in glioma migration, such as transient receptor potential (TRP) calcium channels, which also contribute to changes in Ca^{2+} by modulating the driving force for Ca^{2+} entry as Morrone et al. recently summarized this subject [78].

Microtubes

Osswald et al. reported that astrocytomas but not oligodendrogliomas develop functional multicellular network structures through ultra-long membrane protrusions and that astrocytoma cells use these distinct tumor microtubes as routes for brain invasion, proliferation, and interconnections over long distances [79]. The resulting network facilitates multicellular communication through microtube-associated gap junctions. Confocal microscopy revealed punctate connexin 43 (Cx43) gap junction immunoreactivity, particularly at the tumor microtubes of astrocytoma cells, which was not observed for other connexins. When damage to the network occurs, tumor microtube is used for repair. Moreover, microtube-connected astrocytoma cells, but not those remaining unconnected throughout tumor progression, were protected from cell death inflicted by radiotherapy. Osswald et al. hypothesized that intercellular tumor microtubes can serve as an individual tumor cell to distribute critical elevations of small molecules, such as calcium,

within the large network (Figure 3), achieving nonlethal levels for radiotherapy-induced

cytotoxicity and for intrinsic apoptotic cell death in glioma cells [79]. Neuronal growth-associated

protein 43 (GAP-43) is important for microtube formation and function and drives microtube

dependent tumor cell invasion, proliferation, interconnection, and radioresistance. The

disconnection of astrocytoma networks by disrupting their tumor microtubes may emerge as a new

principle to reduce the treatment resistance of this disease, but clinical applications have not been

introduced [79].

Extracellular MicroRNAs

Over the past decade, there has been increased awareness of the possible role of microRNAs

(miRNAs) in the development of cancer, including glioblastoma [80]. miRNAs are small non-

coding RNAs of approximately 20-23 nucleotides that act as post-transcriptional regulators of gene

expression. miRNAs repress gene expression by binding to complementary sequences in the 3′

untranslated region (3′ UTR) of mRNAs and targeting these molecules for degradation, thereby

preventing their translation, resulting in a decreased level of encoded proteins that affect an array

of cellular processes, e.g., differentiation, proliferation, migration, metabolism, apoptosis and stem

cell maintenance [81]. Aberrant miRNA biogenesis in cancer occurs at different steps during

miRNA maturation [80], and a single miRNA can regulate the expression of many genes.

Depending on its target genes, miRNA can act as a tumor suppressor or tumor promoter. The

accumulation of specific miRNAs in glioblastoma samples from patients and in glioblastoma cell

lines was initially demonstrated a decade ago [82,83]. Since then, numerous reviews have

highlighted the involvement of miRNAs in glioblastoma progression, migration and radio-

chemotherapy resistance and suggested their potential as therapeutic targets [84-90].

Recently, microRNAs have also been localized to the extracellular space, often encapsulated in

secreted extracellular vesicles (EV), nanometer size membrane-enclosed particles formed either from the fusion of an endosome with the plasma membrane (exosomes) or directly from the cell membrane (microvesicles) and released from numerous cell types, including glioblastoma cells, to mold the tumor microenvironment to their advantage (Figure 3) [91-93]. Neighboring or distant recipient normal and tumor cells can take up EVs containing tissue-specific expressed/secreted microRNAs [94]. In a recent review, Rooj et al. discussed the current knowledge regarding the multifaceted roles of both cellular and EV microRNAs in shaping the glioblastoma microenvironment [93]. Glioblastoma EVs are enriched in several oncogenic microRNAs, including miR-21, miR-23a, miR-30a, miR-221 and miR-451 [95]. However, the functions of most of these microRNAs remain unknown. Hoshino and colleagues elegantly demonstrated that tumor-derived EVs are internalized in organ-specific cells, and this process plays an important role in the preparation of the pre-metastatic niche [96]. EVs containing miRNA were also detected in the blood or the cerebrospinal fluid of glioblastoma patients [97]. Thus, microRNAs circulating in complexes with tumor-originated EVs represent a potential source of biomarkers for the early detection and monitoring of tumor responses to treatment and could be utilized for glioblastoma therapy using therapeutic mi-RNA [94]. Arscott et al. showed that irradiation increases the abundance of exosomes released by glioblastoma cell lines and normal astrocytes [98]. These authors showed that exosomes derived from irradiated cells enhanced the migration of recipient cells, and their molecular profiling revealed an abundance of molecules related to signaling pathways important for cell migration, including activation of neurotrophic tyrosine kinase receptor type 1 (TrkA) and FAK signaling [98].

Relationship between cell migration and resistance to pro-apoptotic stimuli

Intracellular signaling pathways involved in the acquisition of resistance to pro-apoptotic

stimuli by migrating glioma cells, including the PTEN/PI3K/Akt/mTOR and NF-kB pathways, were already an important topic more than 10 years ago [31]. These pathways are not all constitutively activated at the same time in all cells in any one glioma. Many inhibitors of these pathways have been developed, and numerous clinical trials have investigated these agents alone or in conjunction with conventional radiotherapy or chemotherapy. Several recent publications have provided an update on the state of EFGR and PI3K/Akt/mTOR inhibitors in clinical trials for glioblastoma [99,100]. So far, targeted therapy directed to central intracellular signal pathways has not yet achieved satisfactory results in glioblastoma. A future perspective for glioblastoma therapy may be the combined inhibition of multiple targets and more tumor-tailored strategies.

As indicated above, potassium channels may be involved both in the migratory processes and resistance to pro-apoptotic stimuli of glioblastoma cells. A volume change and decrease in intracellular K^+ concomitantly occur with the initiation of apoptosis in glioma cells [101]. Kv1.3, expressed at the plasma membrane, is also expressed at the inner membrane of mitochondria, where it is directly inhibited by the proapoptotic protein BAX, resulting in cytochrome c release and initiation of apoptosis [102]. Similar mechanisms are shared by other K^+ channels implicated in apoptosis [103]. Staudacher et al. showed that the small molecule Kv11.1 ligand doxazosin induced concentration-dependent apoptosis of human LNT-229 and U87MG glioma cells, accompanied by cell cycle arrest in G0/G1 [69].

In addition, compared with normal cells, several human cancers exhibit a high mitochondrial membrane potential and low expression of the Kv1.5 channel, which both contribute to the apoptosis resistance of cancer cells, including glioma cells [104]. Closing K^+ channels or decreasing their expression in cancer cells increases the intracellular K^+ concentration, which in turn increases the tonic inhibition that cytosolic K^+ exerts on caspases [103,105].

As indicated above, glioma cells can extend tumor microtubes for brain invasion, proliferation, and interconnection of single cells to a syncytium that is resistant to radiotherapy [79]. Recently Weil et al. evidenced using patient-derived glioblastoma stemlike cell lines (GBMSCs) implanted into the brain of mice that after a surgical lesion GBMSCs increasingly extended tumor microtubes toward the lesion area [106]. In fact, an excessive "healing response" was observed in which tumor cell densities significantly exceeded those of unlesioned brain regions. Moreover, inhibition of tumor microtube formation and function by genetic targeting of growth associated protein-43 robustly suppressed this surgery-induced tumor growth reaction, in contrast to standard postsurgical anti-inflammatory treatment with dexamethasone. After one cycle of TMZ chemotherapy, intra- and intertumoral heterogeneity of tumor microtube formation and interconnection was strongly associated with therapy response: when tumor cells were integrated in tumor microtube networks, they were more likely to resist chemotherapy [106].

New anti-glioblastoma drugs related to migration, invasion and resistance to apoptosis

Targeting glutamate

The multitude of effects of glutamate in glioma biology has supported the rationale for the pharmacological targeting of glutamate receptors and transporters in the treatment of glioblastoma ([107] for review). Using the web site http://www.clinicaltrials.gov/ as a reference, we analyzed the few clinical trials completed with therapies targeting glutamate (see also [108]).

Glutamate release can be inhibited by sulfasalazine, an FDA-approved drug for the treatment of inflammatory bowel disease, through inhibition of the CXC network. This drug may also facilitate death receptor-mediated apoptosis of glioma cells [109]. Sulfasalazine could be considered a

therapeutic agent for glioma patients to reduce seizure activity and control invasive growth in patients, although a preliminary study of ten patients with recurrent glioblastoma indicated lack of clinical efficacy [110] (Table). Yet, small series of 10 patients are insufficient to draw definitive conclusions about the potential therapeutic benefit of any given drug. Accordingly, Huberfeld and Vecht have discussed the potential for single therapeutic agents, such as the CXC system blocker sulfasalazine or histone deacetylase inhibitor valproic acid, to treat both gliomas and associated epilepsy [111]. However, the same year valproic acid was definitively excluded as a useful therapeutic approach after the analysis of survival of a pooled patient cohort (n = 1,869) of four contemporary randomized clinical trials in newly diagnosed glioblastoma patients that received the antiepileptic drug at the start of chemoradiotherapy with temozolomide [112].

Talampanel, an allosteric inhibitor of AMPA receptors, entered phase II clinical trials, and it has been speculated that this drug may provide a survival advantage when used in combination with standard chemoradiotherapy [113,114] (Table). However, Grossman and colleagues challenged their own conclusions one year later [115]. Overall survival of 244 patients with newly diagnosed glioblastoma accrued to three consecutive therapeutic trials conducted by the New Approaches to Brain Tumor Therapy (NABTT) CNS Consortium was significantly higher than is reported with radiation and temozolomide alone [30]. The improvement in survival was evident as early as 6 months after study initiation (94% vs 86% survival, p <0.003) and continued over two years (37%vs 27%, p < 0.02). In each of these trials a novel agent including talampanel for 72 patients was combined with standard radiation and temozolomide [115]. This report documents significant improvements in the survival of patients with newly diagnosed glioblastoma. However, Grossman et al. emphasized that it remains uncertain if these results are attributable to the novel therapies themselves or reflect evolving patterns of care in this patient population [115]. This argues strongly against the overinterpretation of survival data from uncontrolled phase II studies in newly

diagnosed glioblastoma, as has recently been confirmed with the negative outcome of the phase III

ACT IV trial [116].

Memantine, a noncompetitive inhibitor of NMDA receptors approved for dementia treatment, is a

candidate for modulating the effects of glutamate at the infiltrative rim of gliomas [108,117].

Indeed, a phase I factorial trial of temozolomide, memantine, mefloquine, and metformin for post-

radiation therapy glioblastoma is ongoing

(https://clinicaltrials.gov/ct2/show/NCT01430351?term=memantine&cond=glioma&rank=3)

(Table).

The goal of this study is to identify the highest tolerable dose of TMZ in combination with a

mixture of memantine, mefloquine, and/or metformin that can be administered to glioblastoma

patients who have previously undergone radiation and chemotherapy in combination. The safety of

these drug combinations will also be studied

(https://clinicaltrials.gov/ct2/show/NCT01430351?term=memantine&cond=glioma&rank=3).

However, whether such complex trials advance standards of care in glioblastoma remains doubtful,

and exploring the addition of high doses of memantine alone to standard chemoradiotherapy might

have been preferable. Yohay et al. demonstrated that the local polymer-based delivery of riluzole, a

glutamate uptake blocker approved for the treatment of amyotrophic lateral sclerosis, or memantine

improved survival in a rat intracranial glioma model when the polymer wafers were placed at the

time of tumor engraftment [118].

We speculate that normal neurons might be protected against the high glutamate microenvironment

by specific inhibitors of ionotropic receptors but further research is warranted [119,120].

Targeting ion channels

One Cl⁻ channel inhibitor, TM-601, a synthetic version of the peptide chlorotoxin, a small 36 amino

acid neurotoxin isolated from the venom of the giant yellow Israeli scorpion *Leiurus quinquestriatus* [121] that is covalently linked to iodine 131, has completed phase I and II clinical trials for the treatment of anaplastic astrocytomas and glioblastomas through repeated local intracavitary administrations (NCT00591058) [46,122] (Table). Intracavitary-administered 131I-TM-601 is well tolerated, remains highly localized to the treatment site, and preliminarily appears safe for repeated injections. As no major toxicity and no death due to 131I-TM-601 have been reported, the FDA allowed the trial to go to Phase III [123]. An FDA approval has also been obtained to investigate the effect of TM601 in newly diagnosed gliomas. TM601 appears to be stable, is not immunogenic and lacks toxicity in humans upon local administration. Parallel to this trial, the same team has worked on an intravenous injection protocol of this product but this time with an imaging application in perspective [124]. However, to our knowledge no study has been published recently. Interestingly, intracranial injection of 131I-CTX detects brain tumors by gamma-ray scintigram scans in vivo, but also labels the stomach, indicating that the molecular target of chlorotoxin is also expressed in this organ. So far, TM-601 is the only derivative of chlorotoxin for which human clinical studies have been partially published. Besides the clinical trial of 131I-TM-601, many other chlorotoxin applications have been described that may be potentially useful to treat glioma. A large proportion of them rely on the administration of therapeutic agents, cDNA or siRNA to block oncogene expression, thanks to the use of nanoparticles [125,126].

Molecular targets for chlorotoxin include voltage gated chloride channels, calcium-dependent phospholipid-binding protein annexin-2, and matrix metalloproteinase-2 (MMP-2) [127]. Among these targets, MMP-2 has most anti-neoplastic potential [127].

According to Pardo and Stühmer [48] and our previous study [54], inhibition of potassium channels

also has therapeutic potential for glioblastoma patients. Wulf et al. have previously discussed the pharmacological strategies for targeting potassium channels with venom peptides, antibodies or small molecules [128]. The Kv10.1 and Kv11.1 inhibitors have been proposed for the treatment of various types of cancers. Two potent blockers of Kv10.1, the antihistamine, astemizole, and the tricyclic antidepressant, imipramine, have been shown to decrease tumor cell proliferation *in vitro* and, in the case of astemizole, also *in vivo* [129]. Imipramine binds to the intracellular pocket and consequently inhibits the current of Kv10.1 [130]. Desipramine and imipramine have been demonstrated to inhibit PI3K/Akt/mTOR signaling and induce autophagic cell death *in vitro* in murine C6 and human U87MG glioma cells [131,132].

Recently, Sales et al. showed that *in vitro* suppression of the Kv10.1 channel using astemizole or silencing using a short-hairpin RNA expression vector sensitized U87MG glioma cells to temozolomide [133].

Kv11.1 is affected by drugs that cause acquired long QT syndrome [134]. Even if the intracellular channel pore openings of Kv10.1 and Kv11.1 are similar, although not identical, all of the known Kv10.1 blockers are also effective blockers of Kv11.1 and therefore share cardiac safety problems. For these reasons, the antihistamine astemizole was withdrawn from the market in 2000 [135]. Because the risk benefit profile of an anticancer drug is radically different from that of compounds developed for the treatment of benign conditions, the potential for these compounds should be reconsidered for repositioning. Moreover, there are at least three alternative transcripts of Kv11.1, which are differentially expressed in heart versus tumor cells, suggesting the option of selective inhibition of this type of ion channel in tumors while preserving heart function. A specific peptide toxin that can achieve this result has yet to be reported. However, a monoclonal antibody (mAb56) has been developed that specifically blocks Kv10.1 without affecting Kv11.1 or its close relative Kv10.2 [136]. This antibody showed efficacy *in vitro* against human melanoma MDA-MB-435S

and human pancreatic carcinoma AsPC-1 cell lines as well as *in vivo* in nude mice subcutaneously

grafted with these tumor models, but the required doses were high, whereas the reduction of tumor

growth was modest [136]. The experiments were performed in immunodeficient mice, so that the

antibody could, in principle, act exclusively as a channel blocker. Finally, the anticancer compound

roscovitine, a cyclin-dependent kinase inhibitor that has terminated phase II clinical trials for non-

small cell lung cancer [137], is an efficient blocker of Kv11.1, but does not induce arrhythmia,

likely due to its low affinity for closed and inactivated states of the channel.

Staudacher et al. recently identified the Kv11.1 ligand doxazosin as a small molecule compound

that promotes apoptosis and exerts anti-proliferative effects in human glioblastoma cells *in vitro*

[69]. Doxazosin and future derivatives have therefore been proposed as novel options for more

effective glioblastoma treatment [69].

Citalopram (Cipramil®), a selective serotonin reuptake inhibitor used for its antidepressive

activity, acts on Kv1.5 currents as an open-channel blocker [54]. Because both imipramine and

citalopram have been commonly used to treat depression, which commonly occurs in glioma

patients, the European Organization for Research and Treatment of Cancer (EORTC) conducted a

large epidemiological study to investigate the actual benefit that these two drugs may provide for

glioblastoma patients. D'Alessandro et al. demonstrated that i) blockade of the calcium-activated

potassium channel KCa3.1 with TRAM-34, a high affinity blocker of KCa3.1, exhibits good

selectivity for these channels and increases the TMZ antitumor effects, reducing GL-261 glioma

cell migration, invasion and colony forming activity, increasing apoptosis and forcing cells to pass

the G2/M cell cycle phase, likely through cdc2 de-phosphorylation; ii) KCa3.1 silencing

potentiates the inhibitory effect of TMZ on glioma cell viability; iii) the combination of TMZ and

TRAM-34 attenuates the toxic effects of glioma conditioned medium on neuronal cultures through

a microglia-dependent mechanism since the effect is abolished by clodronate-induced microglia

killing; iv) TMZ/TRAM-34 co-treatment increases the number of apoptotic tumor cells and the mean survival time in a syngeneic mouse glioma model (C57BL6 mice implanted with GL261 cells); and v) TMZ/TRAM-34 co-treatment reduces the cell viability of glioblastoma cells and cancer stem cells freshly isolated from patients [138]. Taken together, these data demonstrate that TMZ/TRAM-34 co-treatment affects both glioma cells and infiltrating microglial cells, resulting in an overall reduction of tumor cell progression. Furthermore, calcium activated potassium channel blockade by paxilline, a toxin of the fungus *Penicillium paxilli*, inhibited ionizing radiation-induced migration *in vivo* in an orthotopic model of the human glioblastoma model [139]. Additionally, ophiobolin-A, a sesterterpenoid phytotoxin produced by pathogenic fungi of the genus Bipolaris, induced marked changes in the dynamic organization of the F-actin cytoskeleton and inhibited proliferation and migration of glioblastoma cells, likely by inhibiting conductance Ca(2+)-activated potassium channel activity [140].

Targeting extracellular miRNAs

MicroRNAs are secreted into the extracellular space, at least in part by EV, and are subsequently taken up by normal and cancer cells to sustain and promote tumor growth (Figure 3). Glioblastoma EVs transport several tumor-specific microRNAs; however, the functions of most of these microRNAs remain unknown. A better understanding of the role of secreted microRNAs may unveil a novel mechanism of glioblastoma progression and resistance to current treatments and may provide novel diagnostic and prognostic biomarkers. Rooj et al. suggested that these biomarkers can be used for the development of targeted therapies aimed at improving glioblastoma patient outcomes [93].

Conclusion and perspectives

Glioblastoma is an incurable neoplasm characterized by diffusely infiltrative growth. Given the highly invasive nature of glioblastoma, it remains unlikely that any single therapeutic approach will be able to specifically target and eliminate residual heterogeneous tumor cells. New therapeutic strategies to fight both migrating apoptotic resistant and proliferating glioma cells are necessary to effectively counteract glioblastoma progression. Novel treatment approaches have to integrate the intracellular perspective of the complex intracellular signaling pathways with the microenvironmental orchestral machinery, including connections between heterogeneous tumoral and nontumoral cells, potentially also through channels, microtubes, and extracellular miRNA, generating different messages at different times. New avenues for glioblastoma treatment must not ignore the very specific and peculiar interactions between tumor cells and their host tissue, and the prime targets currently appear to be glutamate- and ion channel-controlled processes.

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FIGURES

FIGURE 1. **The glutamatergic system in glioblastoma.** Glial tumor cells release glutamate by means of the Na^+-independent, Cl^--dependent, cystine/glutamate exchanger system (SXC) transporter. Glutamate released from malignant glioma cells in the extracellular matrix is responsible for seizure induction and, at a higher concentration, neuronal cell death, which is mediated by prolonged activation of neuronal N-methyl-D-aspartate (NMDA) receptors, resulting in Ca^{2+} influx and Ca^{2+}-mediated cell death. This neuronal cell death may facilitate tumor growth. Glutamate also stimulates growth, migration and invasion of glial tumor cells through activation of Ca^{2+}-permeable glutamate AMPA receptors on glioma cells in a paracrine and autocrine manner.

FIGURE 2. **Glioma cell volume and shape regulation: involvement of ion channels.** Cell migration and glioblastoma cell migration in particular is characterized by the polarized cellular and molecular processes that promote the protrusion of the leading edge (local volume increase) and retraction of the trailing edge (local volume reduction). A net outflow of ions and water at the cell trailing edge and a net inflow of ions and water at the cell leading edge underlie these localized hydrodynamic changes. Ion channels and transporters display polarized subcellular distribution. At the trailing edge, calcium entry through calcium-permeable channels can activate calcium-activated potassium channels (KCa) to promote potassium efflux. The opening of chloride (KCl) and potassium channels (Kv) further enhances the outflow of ions Cl^- and K^+, followed by obligatory water efflux through aquaporins. At the leading edge, an assortment of inward ion channels, such as the Na^+/K^+ ATPase and the Na^+-K^+-Cl^- cotransporters (NKCC1) together with aquaporins, regulate ion and water influx. This ion and water influx drives local cell volume increase in the protruding lamellipodium for leading edge extension.

FIGURE 3. **Pathways of intercellular communication in glioblastoma.** Glioblastoma demonstrates intratumoral heterogeneity and contains a population of glioma stem cells (GSC) that contributes to tumor propagation, maintenance and therapeutic resistance.

After secretion, glioblastoma EV microRNAs are taken up by other tumor cells or normal cells in the microenvironment to change their phenotype and support tumor progression.

Structures called microtubes connect tumor cells, enabling these cells to act as a single, organism-like unit. Tumor microtubes facilitate invasion into healthy brain tissue, permitting the spread of toxic molecules, such as Ca^{2+}, which build up during radiotherapy enabling the whole unit to share the burden of toxicity. Furthermore, if tumor tissue is surgically removed, then newly synthesized nuclei donated by tumor cells can travel down the microtube to the cell-free site to form new tumor cells.

We actually thank **Reviewer** for this more than valuable remark.

Reviewer #1: The authors provide an interesting review article on invasion and migration as potential therapeutic targets in glioblastoma.

The authors focus on glutamate, ion channels, tumor microtubes and some signaling pathways. While these topics are of interest, it is unclear why these were selected and other mechanisms or molecules involved in invasion and migration of gliomas were not discussed. Please specify.

"glutamate receptors and cancer":

We focused in the current review on glutamate, ion channels, tumor microtubules and several signaling pathways. While there are a myriad of signaling pathways that influence the migratory behavior of malignant astrocytes (for example see Lefranc et al., JCO 2005), we decided to restrain ourselves on the "glutamate pathway" because we are convinced that impairing this malignant astrocyte-migration avenue of invasion within the brain parenchyma would actually contribute therapeutic benefits to the patients. We detail below why we believe in this avenue to combat malignant gliomas.

We have accordingly added a paragraph in the beginning of **section "glut receptors and cancer"** to satisfactorily respond to **Reviewer's** comment.

Please review the first sentence of the paragraph on microtubes, there seems to be something missing.

Perfomed.

Please discuss PMID 28419303, in which tumor microtubes have been further implicated in treatment resistance.

Performed and ref put.

Incomplete sentence: „An FDA approval has also been obtained to investigate the effect of TM601 in newly diagnosed."
Modified

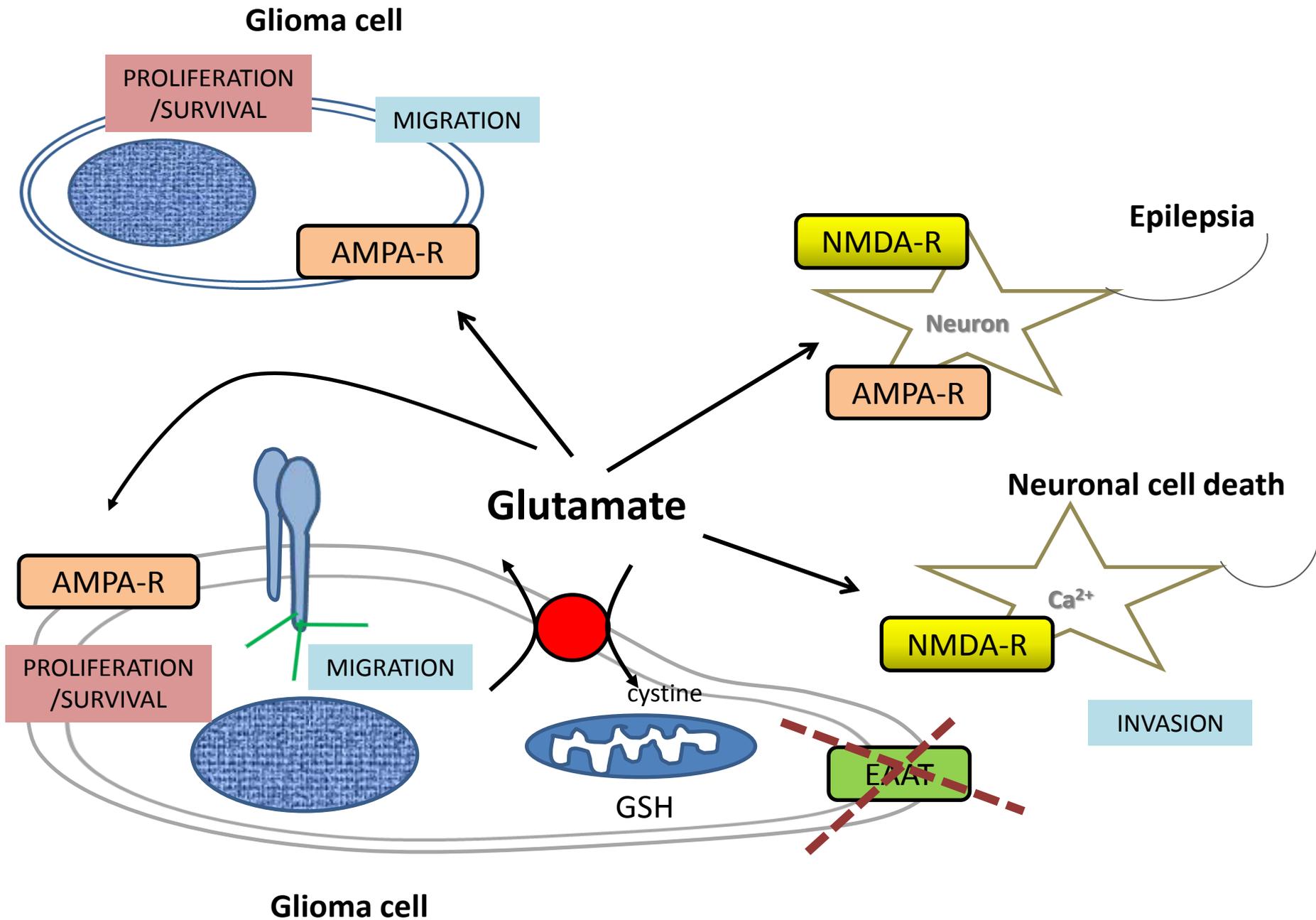A table summarizing the potential treatment approaches would be very useful

Performed
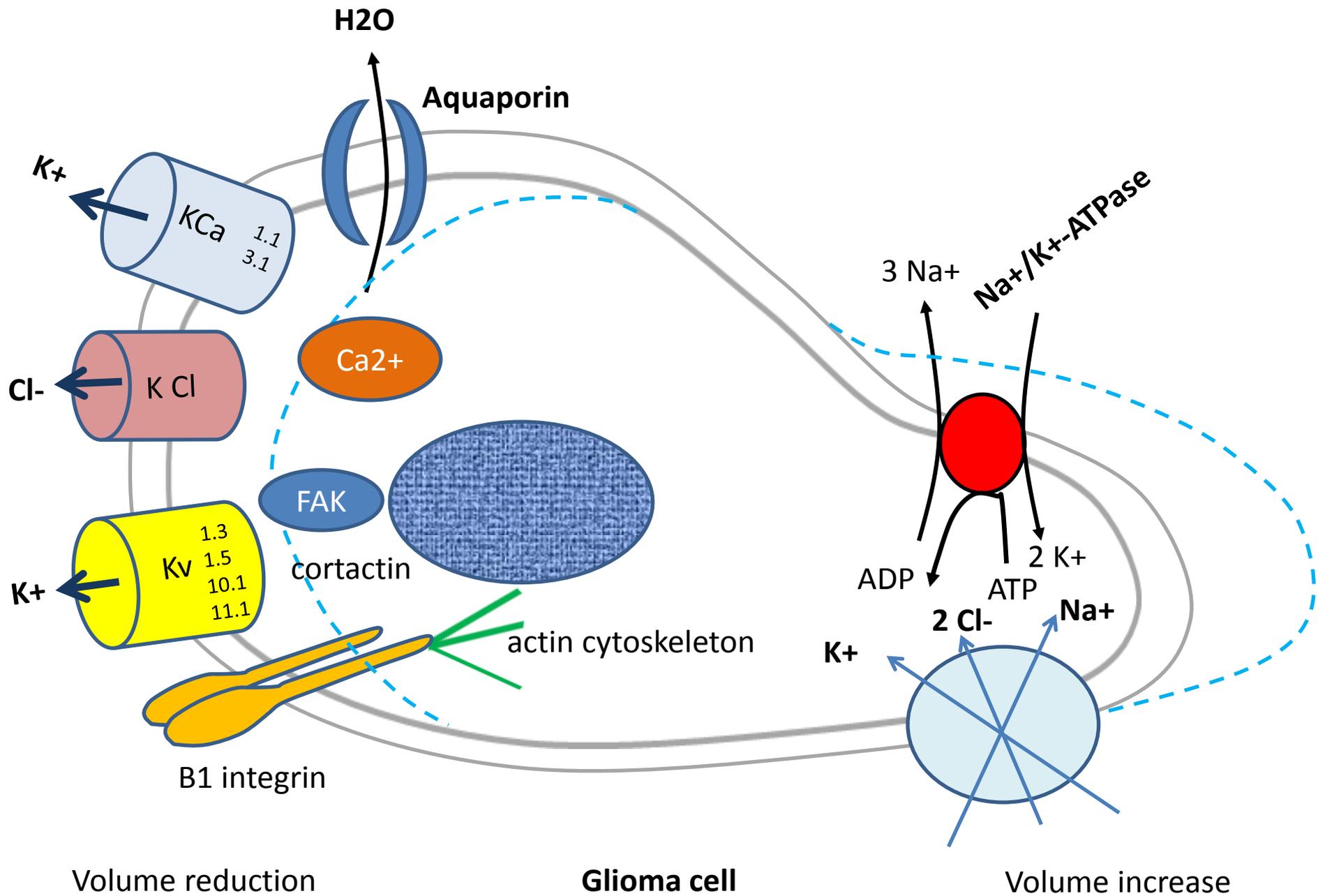
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Erasme

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S www.erasme.ulb.ac.be

Glioma cell

PROLIFERATION/SURVIVAL

MIGRATION

AMPA-R

Glutamate

Epilepsia

NMDA-R

Neuron

AMPA-R

AMPA-R

PROLIFERATION/SURVIVAL

MIGRATION

cystine

GSH

EAAT

Neuronal cell death

Ca²⁺

NMDA-R

INVASION

Glioma cell

H2O

Aquaporin

K+

KCa   1.1
        3.1

Cl-   K Cl

K+   Kv   1.3
             1.5
             10.1
             11.1

Ca2+

FAK

cortactin

actin cytoskeleton

B1 integrin

3 Na+   **Na+/K+-ATPase**

ADP   ATP   2 K+

2 Cl-   **Na+**

K+

Volume reduction   **Glioma cell**   Volume increase

Glioma stem cell

Neuron

Angiogenesis

Extracellular matrix

ions

glutamate

cystine

Glioma cell

Intracellular Signaling pathways

Cx43

vesicles

DNA
mRNA
microRNA

microtubes

Ca^{2+}

Normal cell

New glioma cell formation

Glioma cell

Table: Potential treatment approaches in clinical study

Targets	Compounds	Indications	Study	Ref.
Glutamate release by CXC network	Sulfasalazine	Recurrent glioblastoma	Phase I/II	110
AMPA receptors	Talampanel	Newly diagnosed glioblastoma	Phase II in association with radio- and temozolomide	113, 114, 115
NMDA receptors	Memantine	Glioblastoma	Phase I in association with temozolomide	On going
Cl- channel	TM-601	Anaplastic astrocytoma and glioblastoma	Phases I/II and III intracavitary	46, 122