Published in final edited form as:

Curr Opin Pharmacol. 2018 August ; 41: 79–88. doi:10.1016/j.coph.2018.05.002.

Novel approach to temozolomide resistance in malignant glioma: connexin43-directed therapeutics

Christina L Grek¹, Zhi Sheng^{2,3,4,5}, Christian C Naus⁶, Wun Chey Sin⁶, Robert G Gourdie^{2,3,7,8}, and Gautam G Ghatnekar¹

¹FirstString Research, Inc., Mount Pleasant, SC, USA

²Virginia Tech Carilion Research Institute, Roanoke, VA, USA

³Faculty of Health Science, Virginia Tech, Blacksburg, VA, USA

⁴Department of Biological Sciences and Pathobiology, Virginia-Maryland College of Veterinary Medicine, Virginia Tech, Blacksburg, VA, USA

⁵Department of Internal Medicine, Virginia Tech Carilion School of Medicine, Roanoke, VA, USA

⁶Department of Cellular and Physiological Science, Life Sciences Institute, University of British Columbia, Vancouver, Canada

⁷Virginia Tech-Wake Forest University School of Biomedical Engineering and Sciences, Blacksburg, VA, USA

⁸Department of Emergency Medicine, Virginia Tech Carilion School of Medicine, Roanoke, VA, USA

Abstract

Resistance of malignant glioma, including glioblastoma (GBM), to the chemotherapeutic temozolomide (TMZ) remains a key obstacle in treatment strategies. The gap junction protein connexin43 (Cx43) has complex roles in the establishment, progression, and persistence of malignant glioma. Recent findings demonstrate that connexins play an important role in the microenvironment of malignant glioma and that Cx43 is capable of conferring chemotherapeutic resistance to GBM cells. Carboxyl-terminal Cx43 peptidomimetics show therapeutic promise in overcoming TMZ resistance via mechanisms that may include modulating junctional activity between tumor cells and peritumoral cells and/or downstream molecular signaling events mediated by Cx43 protein binding. High levels of intra-tumor and inter-tumor heterogeneity make it difficult to clearly define specific populations for Cx43-targeted therapy; hence, development of *in vitro* models that better mimic the microenvironment of malignant glioma, and the incorporation of patient-derived stem cells, could provide opportunities for patient-specific drug screening. This review summarizes recent advances in understanding the roles of Cx43 in malignant glioma, with

Corresponding author: Grek, Christina L (grek@firststringresearch.com).

Conflict of interest statement

Drs. Gautam Ghatnekar and Robert Gourdie are coinventors of aCT1 and cofounded FirstString Research. FirstString Research has an exclusive, worldwide license for all fields of use for aCT1. Dr. Ghatnekar is currently president and CEO of FirstString and has company stock options. Dr. Grek is an employee of FirstString Research and has company stock options. Dr. Gourdie is a member of the Scientific Advisory Board of FirstString Research and has stock options issued by the company.

a special focus on tumor microenvironment, TMZ resistance, and therapeutic opportunity offered by Cx43 peptidomimetics.

Introduction

Malignant glioma includes grade II to IV glioma, with grade IV glioblastoma (GBM) as the most common, aggressive, and lethal primary CNS malignant tumor in the US [1]. Malignant gliomas are thought to arise from non-neuronal glial cells that rapidly proliferate into solid tumors characterized by intra and inter-tumoral heterogeneity, resistance to conventional treatments, and poor prognosis [2]. The current treatment regimen for malignant gliomas, particularly GBM, includes surgical resection followed by radiation and cytotoxic chemotherapy. These conventional treatments retain an alarmingly poor prognosis, prolonging median survival from just 12.2 to 14.6 months [3]. Failure to generate more effective treatment strategies is in part due to the infiltrative nature of malignant glioma cells, which hinders complete surgical resection, and cellular heterogeneity within tumors, with a sub-population of self-renewing tumorigenic glioma stem cells (GSCs). Self-renewing GSCs that escape surgical resection are resistant to chemotherapeutic alkylating agents including temozolomide (TMZ), thereby fueling tumor recurrence. TMZ resistance can be conferred by the methylguanine-DNA methyltransferase (MGMT) enzyme, which repairs mutagenic effects of TMZ on DNA. As a result, tumor cells expressing high levels of MGMT display significant resistance to TMZ therapy [4]. However, sensitivity of GBM cells to TMZ may also be independent of MGMT expression [5,6^{**}], highlighting the challenges presented by cellular heterogeneity in malignant glioma and the need for novel approaches in eliminating such refractory tumor cell populations to successfully treat this disease and prevent recurrence.

Cellular communication is vital in numerous processes critical for biological homeostasis. The family of connexin proteins plays essential roles in cell survival, proliferation, differentiation and patterning, and by default, disease [7]. Connexins are tetra-span plasma membrane proteins that are the key structural component of cellular channels, gap junctions (GJs), formed between neighboring cells. GJs have critical roles in cell–cell communication [gap junctional-mediated intercellular communication (GJIC)] through the passive intercellular passage of small molecules, second messengers, ions, microRNAs and electrical signals that serve to maintain tissue homeostasis [8]. Similarly, connexin channels facilitate paracrine communication between the cytosol and extracellular environment via unopposed membrane hemichannels located at the plasma membrane surface [9,10]. Hemichannels on different cells can form heterotypic or homotypic GJ channels, depending on connexin isotype, that may result in unique and specific biophysical properties rendering preference with regard to passaging molecules. An added layer of complexity is afforded through channel-independent mechanisms via the stabilization or destabilization of multiprotein complexes mediated through protein binding sites on the connexin protein. Connexin43 (Cx43) is the most ubiquitous connexin and in addition to having critical regulatory and developmental roles in many tissues, has been implicated as a driver of tumor invasion [11^{**}], a marker of tumor progression [12], and an inducer of TMZ resistance in GBM cells [6^{**},13–15,16^{**}]. Furthermore, the expression and function of connexins in the

microenvironment of malignant glioma is an evolving field, where recent studies investigating the function of Cx43 in the tumor microenvironment reveal that Cx43 in peritumoral cells regulates gliomagenesis, tumor progression, and treatment resistance. As would be anticipated, Cx43 has emerged as a novel target for potential therapeutic modalities aimed at reducing GBM invasiveness, proliferation, and lethality. This review discusses key reports investigating the role of Cx43 in the tumor microenvironment, in chemotherapeutic resistance, and in developing novel therapeutic strategies using Cx43 peptidomimetics aimed at modulating Cx43 as an adjuvant therapeutic approach.

Connexin43 expression in malignant glioma

A literature review of the role of Cx43 in malignant glioma reveals seemingly paradoxical reports citing Cx43 as both a tumor suppressor and a promoter of tumor progression [12]. A tumor suppressive function of Cx43 in malignant glioma was first identified in a rat C6 malignant glioma model where over-expression of Cx43 in C6 cells enhanced GJ function [17] and significantly suppressed cell growth and tumor formation in rats [18]. These results were further supported by the observation that cell death was enforced by ectopically expressed Cx43 in Cx43-low U251 and T98G human GBM cell lines [19]. Analysis of Cx43 protein in 18 glioma patient specimens, including 6 GBM tumors revealed an inverse correlation between Cx43 levels and tumor grade, with 6 GBM samples entirely lacking Cx43 expression [20]. While these data are consistent with the hypothesis that Cx43 is a tumor suppressor, ongoing research has revealed novel functions of Cx43 in GBM disease progression and therapeutic responses. In three studies using a much larger cohort of GBM patients (16, 37, and 74 specimens, respectively), percentages of patients expressing Cx43 were 62.2%, 62.5%, and 77%, respectively [13,21*,22]. A report by Cottin and colleagues further determined that more than 10% of GBM tumors (9 out of 74) displayed strong Cx43 immunostaining [22]. This study also highlights the importance of functional GJs and GJIC in anti-cancer cytotoxic therapies that rely on GJ-mediated bystander effect. For example, dibutyryl cyclic adenosine monophosphate was found to sensitize medulloblastoma cells to TMZ, possibly through increasing the expression of Cx43 and activating a Cx43-induced bystander effect [23]. We recently found that Cx43 levels were notably high in 2 out of 9 GBM cell lines and 4 out of 6 primary GBM samples [6**]. This variability in Cx43 expression levels suggests that this GJ protein plays divergent roles in GBMs. This may also extend to differential expression of other connexins by GBM cells [24]. Lathia and colleagues reported that the ratio of Cx46 to Cx43 was elevated in GSCs, whereas following differentiation into non-CSC GBM cells, Cx46 is reduced and Cx43 is increased. Furthermore, characterization of 10 GSCs lines derived from established cell lines, xenografts, and freshly dissected patient tumor tissues indicated variation in self-renewal capabilities, gene expression profiles, and chemotherapeutic susceptibility [16**]. Recent reports also emphasize the importance of Cx43 subcellular localization and how these findings may translate to recently reported *in vitro* findings [21*]. Given that expression and localization of Cx43 in the nucleus and cytoplasm has been shown to reduce the growth of human GBM cells without a corresponding change in GJIC [19], further investigation into the molecular interactions and signaling pathways that may be impacted by Cx43 subcellular localization and what roles these may play in glioma progression and chemotherapeutic

resistance is warranted. The heterogeneous nature of GBM, and the importance of both channel-dependent and channel-independent mechanisms in driving cancer cell growth, migration and apoptosis may offer an explanation to conflicting reports regarding connexin expression profiles in GBM, but also highlight the complexities associated with defining therapeutic strategies.

Connexin43 channels in the glioma microenvironment

The major cell types that constitute the glioma microenvironment include reactive astrocytes, activated microglia, neurons, and endothelial cells, where a glial scar consisting of reactive astrocytes is usually observed to encase gliomas [25] (Figure 1). Recent studies highlight the complexities of the tumor microenvironment in mediating the etiology, progression and invasion of GBM tumors, where three major niches (hypoxic core, invasive niche at the tumor border, and perivascular niche containing blood vessels) witness distinct subsets of cellular interactions [26,27]. Furthermore, due to the production of tumor cell-derived and host stromal cell-derived growth factors, cytokines and matrix proteins, the tumor microenvironment represents a dynamic niche for tissue remodeling, where glioma cells can hijack molecular programs involved in normal tissue development, including immune signaling pathways, to promote their own survival and expansion [25,28,29].

The tumor microenvironment not only determines GBM cell behavior but can mediate resistance to chemotherapeutics, including TMZ [30*]. In the microenvironment, Cx43-mediated GJIC is expected to occur between tumor cells, between astrocytes, and heterocellularly between tumor cells and astrocytes (Figure 1). Interestingly, the physical interaction (as well as the GJIC) that occurs between astrocytes and tumor cells may play a role in chemotherapeutic resistance of GBM cells [30*]. Cx43 also mediates cellular signaling as a unitary channel/hemichannel that permits paracrine communication between the cytosol and the extracellular environment [9,10]. Hemichannel function may overlap with that of exosomes [31], small extracellular vesicles that have been recently shown to facilitate metastasis by transporting appropriate integrins to target organs [32]. A recent report based on a glioma-astrocyte co-culture system suggests that GJs and exosomes each have a role in the facilitation of tumor invasion [33]. The finding that Cx43 is detected in exosomes further supports the need for in-depth investigation into the role of Cx43 in the tumor microenvironment [34].

Overexpression of Cx43 in malignant gliomas has been reported to promote migration in a channel-dependent manner, especially in the presence of normal stromal cells such as astrocytes [35–37]. Indeed, it has been recently shown that Cx43 forms a unique channel between tumor cells and astrocytes that allows for the exchange of microRNAs (miRNAs), which are small, non-coding RNA molecules that often regulate several protein targets and have been demonstrated to reprogram normal stromal cells [33]. In particular, this has been highlighted by the identification that miR-5096 is able to enhance the invasiveness of malignant glioma cells in a Cx43-dependent manner by reprogramming the astrocytes to alter the microenvironment [33,38]. Additionally, in GBM cells the transfer of miRNAs through exosomes or GJIC may play a role in chemotherapeutic resistance [39]. GJIC-mediated delivery of anti-miR-9 from mesenchymal stem cells to GBM cells resulted in

reduced expression of the drug transporter MDR1 and reduced TMZ resistance. Further investigation into the mechanisms that underlie Cx43 channel-mediated miRNA exchange in relation to GBM chemotherapeutic resistance may reveal unique therapeutic opportunities.

Reactive astrocytes are one of the major cell types encountered by tumor cells in the brain and promote cell proliferation even after tumor resection [40,41]. Expression of Cx43 is significantly enhanced in tumor-associated astrocytes especially at the region adjacent to the tumor core [11^{**}]. Recent reports suggest that astrocytic Cx43 may contribute to TMZ resistance in addition to enhancing GBM cell proliferation and migration. In a co-culture system of human astrocytes and tumor cells designed to simulate the tumor microenvironment, siRNA knockdown of astrocytic Cx43 abolished GJIC between glioma cells and astrocyte and increased TMZ-induced apoptosis of GBM cells [42]. Furthermore, in a mouse model consisting of syngeneic intracranial implantation of GL261 malignant glioma cells into Nestin-Cre:Cx43^{fl/fl} mice, in which Cx43 is selectively eliminated in astrocytes, it was demonstrated that reduction of astrocytic Cx43 decreases the invasion of tumor cells from the tumor core in the presence of an intact immune system [11^{**}]. The importance of astrocytic Cx43 in the regulation of GBM resistance and in tumor invasiveness strongly supports the manipulation of Cx43 in the GBM microenvironment as a promising therapeutic strategy in controlling glioma.

Connexin43 expression and TMZ resistance

Following tumor resection, combinatorial chemotherapy using TMZ is a common strategy due to the ability of this drug to cross the blood brain barrier. However, the success of TMZ therapy is hampered by inherent and acquired resistance that may or may not be MGMT dependent. MGMT effectively repairs TMZ-induced DNA damage, thereby limiting TMZ efficacy in GBM patients expressing this gene. However, GBM patients with no or limited expression of MGMT also develop TMZ resistance [5,6^{**}]. Interestingly, in contrast to growth inhibition induced by a reinforced expression of Cx43 in C6 malignant glioma cells [18], several studies have found that ectopically expressing Cx43 in GBM cells confers TMZ resistance. Gielen *et al.* [13] determined that introduction of full-length wild-type Cx43 rendered Cx43-low LN18 and LN229 GBM cells more resistant to TMZ. However, reduced TMZ resistance was observed in cells treated with a Cx43 C-terminal truncation mutant that impacts both channel and non-channel activities of Cx43 by diminishing interactions between Cx43 and C-terminal binding proteins such as Zonula Occludens-1 [54] as well as cytoskeletal proteins including p120 catenin and N-cadherin. Similarly, introduction of Cx43T154A, a dominant negative Cx43 mutation associated with GJs composed of inactive channels [43], did not protect cells from TMZ-associated apoptosis. Indeed, the C-terminal truncated Cx43 decreased GJIC by 50%, while the Cx43T154A mutant completely blocked GJIC in LN229 GBM cells. These findings suggest that an intact C-terminal tail and Cx43-mediated GJIC are necessary for Cx43 to mediate TMZ resistance. In a related study, U251 human GBM cells over-expressing Cx43 became resistant to TMZ, possibly through inhibition of TMZ-induced apoptosis [44]. We and others have similarly reported that GBM cells with high levels of endogenous Cx43 exhibit lower sensitivity to TMZ. Munoz *et al.* [14] found that TMZ treatment increased the expression of Cx43 and GJIC via activation of the 5' regulatory region in U87MG and T98G GBM cells, both of which are resistant to

TMZ. Furthermore, knockdown of Cx43 using a small interfering RNA (siRNA), together with TMZ, substantially induced cell death, suggesting that Cx43 renders U87MG and T98G cells resistant to TMZ. These results were consistent with another study, in which shRNA decrease of Cx43 expression and loss of GJIC sensitized U87MG cells to TMZ [15]. Lai and colleagues exposed Cx43-low/TMZ-sensitive U251 GBM cells to a low dose of TMZ to select a TMZ-resistant U251 clone and subsequently found that TMZ-resistant U251 cells expressed higher levels of Cx43 and, intriguingly, that the MGMT gene showed reactivated expression in the TMZ-resistant clone [45*]. To clarify whether endogenous Cx43 confers TMZ resistance in GBM, we measured levels of Cx43 in 7 MGMT-deficient GBM cell lines and found that lower levels of Cx43 strongly correlated with the increased sensitivity of GBM cells to TMZ when MGMT was not present, suggesting that high levels of Cx43 contribute to MGMT-independent TMZ resistance [6**]. In addition, a GSC line LN229/GSC derived from LN229 GBM cells, exhibited a lower sensitivity to TMZ coinciding with an elevated level of Cx43. Altogether, these studies suggest that Cx43 may be one of the major players conferring MGMT-independent TMZ resistance in GBM. To further elucidate the molecular mechanisms involved, future studies would benefit from the correlation of reported findings with Cx43 subcellular localization, functional GJIC, as well as Cx43 phosphorylation status.

Therapeutic opportunity for connexin43 peptidomimetics

Cx43 peptidomimetics provide a targeted method to selectively modulate the activity of connexin GJs and/or hemichannels as well as the numerous proteins that interact with Cx43 at the cytoplasmic site (Table 1 and Figure 2). We have recently shown that inhibiting Cx43 hemichannel activity using α CT1 (a C-terminal mimetic peptide of Cx43, also referred to as α CT1 or ACT1 in publications; Figure 2) restored TMZ sensitivity in TMZ-resistant/Cx43-high GBM cells including GSCs, and inhibited the growth of LN229/GSC tumors in mice treated with TMZ [6**]. Recent research on Cx43 hemichannels provides some mechanistic insight. Given that Cx43 hemichannels facilitate secretion of certain small molecules such as ATP or glutamate [56], it is possible that inhibition of Cx43 hemichannels by Cx43 peptidomimetics helps retain TMZ in tumor cells, thereby increasing TMZ cytotoxicity. When used in combination with TMZ, targeting Cx43 with this peptide also inhibited GSC cell self-renewal and viability [16**]. Ongoing efforts are geared towards reformulating α CT1 for internal use in brain tumors. Strategies include α CT1 encapsulation in FDA-approved polymers to provide for its controlled-release *in situ* following delivery into brain tumors. Evaluation of a controlled-release α CT1 formulation is currently being evaluated in combination with TMZ chemotherapy in a clinical trial in dogs (FACT-GG trial) with naturally occurring high-grade malignant glioma.

Alternative strategies using cell-penetrating C-terminal mimetic peptides targeting the non-receptor tyrosine kinase c-Src binding domain of Cx43 [(TAT)-PEP-2; (Figure 2)] indicate potential in reversing GSC phenotype and overcoming oncogenic activity through downregulation of the inhibitor of differentiation (ID1), and the transcription factor, SOX-2, and promotion of cadherin switching [52]. In excitotoxic neuronal death models, this same peptide exerted a neuroprotective effect mediated through the reduction of hemichannel activity [53*]. Mechanistic studies using the peptide suggest that inhibition of migration,

invasion, and survival of GSCs may be directly mediated through s-Src associated decreases in FAK phosphorylation and PTEN upregulation [57**]. As c-Src activity has been associated with resistance to anticancer treatments, it is plausible that selective targeting of Cx43 regulated Src activity may be a strategy in overcoming chemotherapeutic resistance. Juxtamembrane peptide 2 (JM2), a peptidomimetic based on the microtubule binding domain of the Cx43 C-terminus similarly inhibits Cx43 hemichannels [55] in addition to impacting migration, invasion, and survival of human GSCs [58], and may have intrinsic anti-cancer properties by promoting microtubule polymerization.

There are a number of additional Cx43 peptidomimetics currently being evaluated for therapeutic potential [7], including Gap26 and Gap27 peptides (Figure 2), mimetics of the Cx43 extracellular loop domain, which rapidly inhibit connexin hemichannels in addition to inhibiting GJ channels after some delay [46,48,59]. The aCT1 peptide is afforded unique therapeutic opportunity from other blockers of Cx43 function as it selectively inhibits hemichannel activity, while maintaining GJs. Interestingly, a related peptide, consisting of the last 9 amino acids of the C-terminal tail of Cx43 has an acute stimulating effect on Cx43 hemichannel opening as a result of a gating effect [47,50], suggesting that aCT1 may have similar effects on acute time scales. At longer time scales (2 hours), aCT1 results in a transition of the hemichannel population into gap junctions. The result is a reduction in the free hemichannel population and therefore activity, and an increase in gap junction accretion and intercellular communication [54]. In injury models, aCT1 has also been found to promote phosphorylation of Cx43 at serine 368 [60] — a post-translational modification associated with hemichannel and GJ channel closure [61,62]. Further evaluation of the phosphorylation status of Cx43 as it relates to TMZ resistance and disease progression may reveal mechanistic details as well as therapeutic opportunity. Peptidomimetic perturbation of the interactive cytoplasmic loop using L2 or Gap19 has also been shown to influence hemichannel function in C6 cells and astrocytes [50,51]. It remains to be determined if these peptides have utility in overcoming chemotherapeutic resistance in various stages or subsets of GBM cells or GSCs. An alternative strategy may involve the use of monoclonal antibodies designed against the extracellular domain of Cx43 to inhibit the connexon structure, alter GJIC and hemichannel activity, inhibit glioma invasion, and/or overcome chemoresistance (discussed in [63*]).

Therapeutic opportunity for peptidomimetics in overcoming TMZ resistance may also be granted by targeting non-channel activities mainly exhibited by the Cx43 C-terminus. The Cx43 C-terminus interacts with a number of signaling molecules including c-Src, protein kinase C, AKT serine/threonine kinase, and mitogen-activated protein kinase to name a few (reviewed in [63*]). These interactions can also affect the phosphorylation status of Cx43 and consequently regulate its degradation, subcellular localization, and channel assembly processes, in addition to potentially mediating GBM resistance to TMZ [64,65]. Independent of channel activity, we have also found that aCT1 sensitizes GBM to TMZ in association with suppression of AKT activity [6**]. These data suggest a possible role for the PI3K/AKT/mTOR signaling pathway in the molecular mechanism of aCT1 in the modulation of TMZ sensitivity. Ongoing studies suggest that peptidomimetic-mediated destabilization of Cx43 protein complexes have a role in abrogating signaling pathways that contribute to TMZ resistance. What is clear is that proteomic interactions and post-

translational interactions change activities of both Cx43 and other signaling molecules. Taken together, the data indicate that Cx43 may confer TMZ resistance in GBM via both channel-dependent and channel-independent mechanisms (Figure 3). Further investigation into the underlying mechanisms by which Cx43 inter-molecular and intramolecular interactions may impact channel-dependent and channel-independent signaling is necessary to optimize therapeutic strategy.

Conclusions and future prospects

There has been limited clinical success in the development of therapeutics for the treatment of malignant glioma including GBM. Pharmacologically targeting Cx43 has emerged as viable adjuvant therapy aimed at preventing tumor invasion and recurrence after primary tumor resection, in addition to overcoming intrinsic or acquired TMZ resistance. The delivery of Cx43 activity targeting compounds to brain tumors pose important challenges that will need to be overcome to realize this potential. Interestingly, cell penetration sequences similar to those incorporated in a number of the peptides listed in Table 1 have shown facility for crossing the blood brain barrier [51,66]. Use of drug delivery vehicles including exosomal vectors may also assist in this key task [67]. Further challenges are afforded by the complexities associated with the tumor microenvironment, cellular heterogeneity and evolving evidence that TMZ resistance is mediated by multiple molecular pathways unrelated to the expression of MGMT. Current investigations highlight the role of Cx43 in providing a permissive niche for tumor invasion and channel-mediated acquired resistance in GBM. Thus, development of systems that better mimic the tumor microenvironment *in vitro* would provide the opportunity to develop an experimental platform with potential for patient-specific drug screening. Aspects of such culture models might include recapitulation of the tissue architecture, pertinent cell types (e.g., GSCs) and inflammatory milieu that characterize tumors *in vivo*. The incorporation of TMZ resistant patient-derived GBM tumor tissues, in addition to methods to evaluate the functional status of Cx43 gap junctions in such models, would aid in research defining the channel-dependent and channel-independent roles of Cx43 in GBM therapeutic resistance. Furthermore, the ability to grow a ‘brain tumor in a dish’ [68,69] from specific individuals with the disease could aid in the development of personalized-medicine treatments that enable clinical assessment of whether a given patient is likely to respond to Cx43-targeting adjuvant therapy.

Acknowledgements

We thank Dr. L. Leybaert, Ghent University, for his review of the manuscript.

Funding

This work was supported by the National Institutes of Health 1R43 CA195937 to FirstString Research, RO1 HL56728 to RGG and R21 CA216768 to ZS; and funding from the Canadian Institutes of Health Research, and the Canadian Cancer Society to CCN and WCS. CCN holds a Canada Research Chair.

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Figure 1.

Potential sites of action by Cx43 targeting peptides. Cx43 intercellular gap junction channels (homotypic or heterotypic; green channels) exist between normal astrocytes that are essential for homeostasis, and between glioma and astrocytes in glioma integration into brain parenchyma. Potential therapeutic opportunity is offered through targeting Cx43 channels that mediate signaling between (1) reactive astrocytes and glioma cells; (2) reactive astrocytes; (3) glioma cells; (4) glioma cells and endothelial cells. Consideration must be taken with regards to the disruption of junctional interaction between (5) normal astrocytes; (6) astrocytes and endothelial cells; (7) astrocytes and neurons. Cx43 hemichannels (red channels) may also have a role in the glioma microenvironment, such as those on glioma, microglia and reactive astrocytes which are expected to release cytokines and chemokines that promote tumor expansion.

Figure 2. Schematic of Cx43. Cx43 peptidomimetics differentially target hemichannel and/or gap junction channel activity, and may also disrupt proteinbinding sites in the Cx43 C-terminus.

Figure 3.

Modes of action of Cx43 in TMZ resistance. Temozolomide (TMZ) is an alkylating agent used in the treatment of malignant glioma. TMZ induces DNA methylation of guanine at O6 position (O6-MG). O6-MG incorrectly pairs with thymine and triggers the mismatch repair (MMR) system leading to DNA strand breaks, the arrest of cell cycle and apoptosis. Cx43-mediated TMZ resistance may occur via the export of TMZ out of cells by Cx43 hemichannels or via Cx43 mediated signaling. See text for additional details.

Table 1

Connexin43 peptidomimetics.

Name	Properties	Reference
Gap 26	Inhibits hemichannels and on longer timescales inhibits gap junctions	[46,47]
Gap 27	Inhibits hemichannels and on longer timescales inhibits gap junctions	[48]
peptide5	Low concentrations prevent hemichannel opening only; higher concentrations also inhibit gap junctions	[49]
L2	Inhibits Cx43 hemichannels	[50]
Gap19	Inhibits Cx43 hemichannels	[51]
PEP-2	Inhibits c-Src interaction and Cx43 hemichannels	[52,53*]
aCT1	Promotes transition of Cx43 hemichannels into GJs; corresponding reduction in hemichannel activity and increases in GJIC	[54]
JM2	Inhibits Cx43 hemichannels and gap junctions. Promotes microtubule polymerization	[55]