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Role of Cl^- channels in primary brain tumour

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ABSTRACT

There is tight interplay between Ca^{2+} and Cl^- flux that can influence brain tumour proliferation, migration and invasion. Glioma is the predominant malignant primary brain tumour, accounting for ~80% of all cases. Voltage-gated Cl^- channel family (ClC) proteins and Cl^- intracellular channel (CLIC) proteins are drastically over-expressed in glioma, and are associated with enhanced cell proliferation, migration and invasion. Ca^{2+} also plays fundamental roles in the phenomenon. Ca^{2+}-activated Cl^- channels (CaCC) such as TMEM16A and bestrophin-1 are involved in glioma formation and assist Ca^{2+} movement from intracellular stores to the plasma membrane. Additionally, the transient receptor protein (TRP) channel TRPC1 can induce activation of ClC-3 by increasing intracellular Ca^{2+}concentrations and activating Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). Therefore, Ca^{2+} and Cl^-currents can concurrently mediate brain tumour cellular functions. Glioma also expresses volume regulated anion channels (VRACs), which are responsible for the swelling-induced Cl^- current, $I_{Cl,swell}$. This current enables glioma cells to perform regulatory volume decrease (RVD) as a survivability mechanism in response to hypoxic conditions within the tumour microenvironment. RVD can also be exploited by glioma for invasion and migration. Effective treatment for glioma is challenging, which can be in part due to prolonged chemotherapy leading to mutations in genes associated with multi-drug resistances (MRP1, Bcl-2, and ABC family). Thus, a potential therapeutic strategy for treatment of glioma can be through the inhibition of selected Cl^- channels.

1. Introduction

Primary brain tumours can be classified into 120 types, divided into benign and malignant tumours. Classification is based on degree of malignancy, location, and cell type of origin. Glioma, which arises from glial cells, represents the predominant malignant primary brain tumour.

Abbreviations: ClC, chloride channel family; CLICc, hloride intracellular channel family; CaCC, ca^{2+}-activated Cl^- channel; K_{Ca}, Ca^{2+}-activated potassium channel; VGCC, voltage-gated calcium channel; TRP, Transient receptor protein; CaMKII, Ca^{2+}/calmodulin-dependent Protein kinase II; VRAC, volume regulated anion channel; RVD, regulatory volume decrease; VSOR, volume sensitive outward-rectifying; ORCC, outward-rectifying Cl^- channel; VSOAC, volume sensitive organic osmolyte/anion channels; CFTR, cystic fibrosis transmembrane conductance regulator; NBD, nuclear binding domain; WHO, World Health Organization; GBM, glioblastoma multiforme; GABA, gamma aminobutyric Acid; $I_{cl,\ swell}$, swelling-induced chloride current; RTK, receptor tyrosine kinase; BK, big-conductance potassium channel; SOCE, store operated Ca^{2+} entry; CRAC, Ca^{2+} release-activated channel; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; IP_3R, inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor; ATP, adenosine triphosphate; STIM1, stromal interaction molecule; RPE, retinal pigment epithelium; Best1, betrophin1; PMC, pre-mitotic condensation; CLCA, chloride chanel regulator; LRRC8A, leucine-rich repeat containing 8A; DCPIB, 4-[(2-Butyl-6,7-dichloro-2-cyclopentyl-2,3-dihydro-1-oxo-1H-inden-5-yl)oxy]butanoic acid; PLC, phospholipase C; CHOP, C/EBP homologous protein; TNFα, tumour necrosis factor α; NKCC, Na-K-Cl Cotransporter; MMP, matrix metalloproteinase; P-gp, phosphoglycoprotein; DIDS, 4,4′-Diisothiocyano-2,2′-stilbenedisulfonic acid; NPPB, 5-Nitro-2-(3-phenylpropylamino)benzoic acid; TSA, trichostatin; DIOA, dihydroindenyloxyacetic; TMZ, temozolomide; Ctx, chlorotoxin; TEA, tetraethylammonium chloride; MDR, multi-drug resistance; ABC, ATP-binding cassette; MRP, multi-drug resistance protein; MVP, major vault protein; GSC, glioblastoma-like stem cells; NSC, neuronal stem cells; CSC, cancer stem cells; EV, extracellular vesicle

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In 2016, WHO made amendments to classify glioma based on diagnostic testing and treatment procedures [1]. Currently, glioma is categorized into four degrees of malignancies: low grade (Grades I-II) and high grade (Grades III-IV). These grades include astrocytoma, oligodendroglioma and ependymoma. Among high-grade glioma, 25% are anaplastic astrocytoma (Grade III) and 75% are glioblastoma (GBM) (Grade IV) [2]. GBM is exceptionally aggressive in its proliferation, migration and invasion [3]. Despite heterogeneity in GBM presentation, morphological characteristics such as necrosis and vasculature defects allow for more reliable diagnosis [4]. Histological diagnosis can be further improved by the analysis of genetic mutations and biomarkers [5]. However, GBM is highly resilient to conventional cancer therapy and is typically a terminal disease [5]. Patients display low life expectancy (~1.5 years following diagnosis) even with aggressive treatment [6]. Thus, there is an urgent need for novel drug targets for the treatment of GBM [5].

Glioblastoma-like stem cells (GSC), are glioma cells that carry the self-renewal properties of neuronal stem cells (NSC) and contribute to the highly invasive nature of GBM. Compared to NSCs, GSCs are enriched with ion channels including voltage-gated Ca^{2+} channels and voltage-gated Cl^- channels which, when pharmacologically inhibited, reduced GSC viability across all cell lines [7].

In the brain, Cl^- channels have various physiological roles. The voltage-gated ClC channel family, which is comprised of eight members, is involved in membrane potential stabilization, epithelial transport, pH regulation and vesicle ion homeostasis [8]. Cl^- intracellular channel protein (CLIC), a six-membered Cl^- channel, is involved in several biological processes such as membrane trafficking and endosomal sorting [9]. Ligand-gated Cl^- channels such as GABA- and glycine-gated Cl^- channels coordinate growth of neuronal stem cells via neurotransmission [3]. Ca^{2+}-activated Cl^- channels (CaCCs) including Ano1 (TMEM16A) and Bestrophin-1 are closely involved with cellular Ca^{2+} homeostasis as well as cell volume regulation [10,11].

Dysregulation of these Cl^- channels has also been associated with a number of brain pathologies such as epilepsy, mental retardation, and hyperekplexia [8]. One trait of GBM is the involvement of aberrant Cl^- channel activity, which can enhance tumour proliferation, migration and invasion. Thus, channelopathy is an underlying cause, caused by overexpression and/or post-translational modifications of Cl^- channels [8,9]. Moreover, the activity of Cl^- channels is heavily influenced by Ca^{2+} homeostasis via TRP and Ca^{2+}-activated K^+ (K_{Ca}) channels [8,10,11]. Ca^{2+} and Cl^- channel activity also act synergistically to induce swelling-induced Cl^- currents ($I_{Cl,swell}$), which upregulate pro-survival signalling pathways and drive regulatory volume decrease (RVD) for glioma migration and invasion [12,13].

Non-glioma malignant primary brain tumours include primary cerebral lymphoma, pineal and pituitary tumours, and acoustic neuroma [2]. Nonetheless, these tumours are relatively rare compared to glioma, and there is insufficient literature documenting the involvement of Cl^- channels. This article provides an overview of the mechanistic interplay between Ca^{2+} and Cl^-, noting the involvement of relevant Ca^{2+} and Cl^- channels in glioma cellular functions.

2. Calcium & chloride channels involved in glioma functions

Ion channels have critical roles both physiologically and pathologically, including cancer. Glioma relies on migration to invade surrounding brain tissue [12]. A key proponent of invasion is cell volume change. Shrinkage can assist glioma in traversing narrow regions [2]. Osmotically-induced volume changes primarily depend on Ca^{2+} and Cl^- fluxes through various ion channels [2]. Voltage-Gated Calcium Channels (VGCCs), transient receptor potential (TRP), and Ca^{2+}-activated K^+ (K_{Ca}) channels play an important role in Ca^{2+} homeostasis in GBM. In addition, the most notable Cl^- channels involved include Ca^{2+}-activated Cl^- (CaCC) channels, as well as members of the voltage-gated Cl^- channel (ClC) family that contribute to the swelling-induced Cl^- current $I_{Cl,swell}$.

2.1. Calcium channels and calcium homeostasis

2.1.1. Voltage-gated calcium channels (VGCCs)

VGCC includes low-voltage activated (T-type) and high-voltage activated (L, N, P/Q, and R-type) Ca^{2+} channels, among which P/Q-type, N-type, and T-type channels are abundantly expressed in brain cells. VGCCs mediate Ca^{2+} oscillations in primary brain tumours and are involved in glioma aggressiveness and malignancy [18]. For example, T-type VGCC blocker mibefradil decreased GBM cell proliferation and migration, induced GBM cell apoptosis *in vitro* [19], and suppressed GBM growth in a murine xenograft model [20]. Therefore, recovery of the intracellular Ca^{2+} balance by suppressing VGCCs through inhibitors or other regulators may provide an effective approach for the treatment of primary brain tumours.

2.1.2. Transient potential receptor (TRP) channels

Within this superfamily of non-selective cation channels, TRPM7 (a member in the melastatin subfamily) has been strongly suggested to influence glioma cellular Ca^{2+} concentrations, and promote survival and motility [17–19]. Our group and others have reported that TRPM7 is upregulated in the U87 and U251 GBM cell lines [17–19]. Inhibition of TRPM7 by carvacrol [21] and xyloketal B [19] induced apoptosis, prevented migration and invasion, and downregulated RAS/MEK/MAPK and PI3K/ATK pathways. In contrast, potentiation of TRPM7 by naltriben increased GBM migration and invasion, and upregulated the ERK pathway [23]. The current evidence strongly indicates that TRPM7 is involved in GBM functions (i.e. suppressed with TRPM7 inhibition; enhanced with TRPM7 potentiation). TRPM7 is speculated to act upstream of the aforementioned pro-survival pathways through activation of receptor threonine kinases (RTKs) and the PLC pathway [17,18,20]. In A172 glioma cells, elevated TRPM7 expression promotes cell proliferation and migration through activation of the Notch and JAK2/STAT3 pathways [24]. TRPM7 also contributes to glioma migration by mediating Ca^{2+} entry, which can activate proteins involved in the modulation of cell adhesion dynamics [25]. In addition, TRPM7 triggered Ca^{2+} sparks that promoted the formation of invadosomes [25]. TRPM7 activity, and consequently intracellular [Ca^{2+}], decreased in response to an increase in intracellular Cl^- concentration [26]. This suggests that Cl^- flux in response to hypertonic conditions can indirectly inhibit Ca^{2+} influx by downregulating TRPM7.

2.1.3. Calcium-activated potassium (KCa) channels

Glial cell proliferation is partly mediated by membrane conductance variations via Ca^{2+} and K^+ flux [27]. Moreover, Ca^{2+}-activated K^+-channels (K_{Ca}) can influence glioma cell migration and metastasis [28]. Big conductance K_{Ca} channels (BK) are upregulated in GBM due to a mutation in the *hSlo* gene and show an increase in sensitivity to Ca^{2+} [29]. The overexpressed BK channel $K_{Ca}1.1$ was shown to mediate growth in Muller glial cells and I321 N astrocytoma cells [29]. $K_{Ca}1.1$ is co-localized with a member of the Cl^- channel (ClC) family (ClC-3), and this interaction was associated with invadopodium formation and enhanced glioma invasiveness [30,33]. Upregulated expression of the intermediate conductance $K_{Ca}3.1$(IK) was reported in U87, which promote increased Ca^{2+} entry through TRP channel [31]. This subsequently enhanced Ca^{2+} oscillations, which underlies the motility mechanisms responsible for glioma migration [32].

2.1.4. Role of Ca^{2+} homeostasis in glioma

Maintaining Ca^{2+} homeostasis involves Ca^{2+} pumps, channels, and exchangers which mediate the movement of Ca^{2+} between the endoplasmic reticulum (ER) or sarcoplasmic reticulum (SR), the cytosol, and extracellular space [29,30]. Ca^{2+} dysregulation can affect many signalling pathways in glioma, including those involved in cell proliferation, migration, invasion, and apoptosis [31,23].

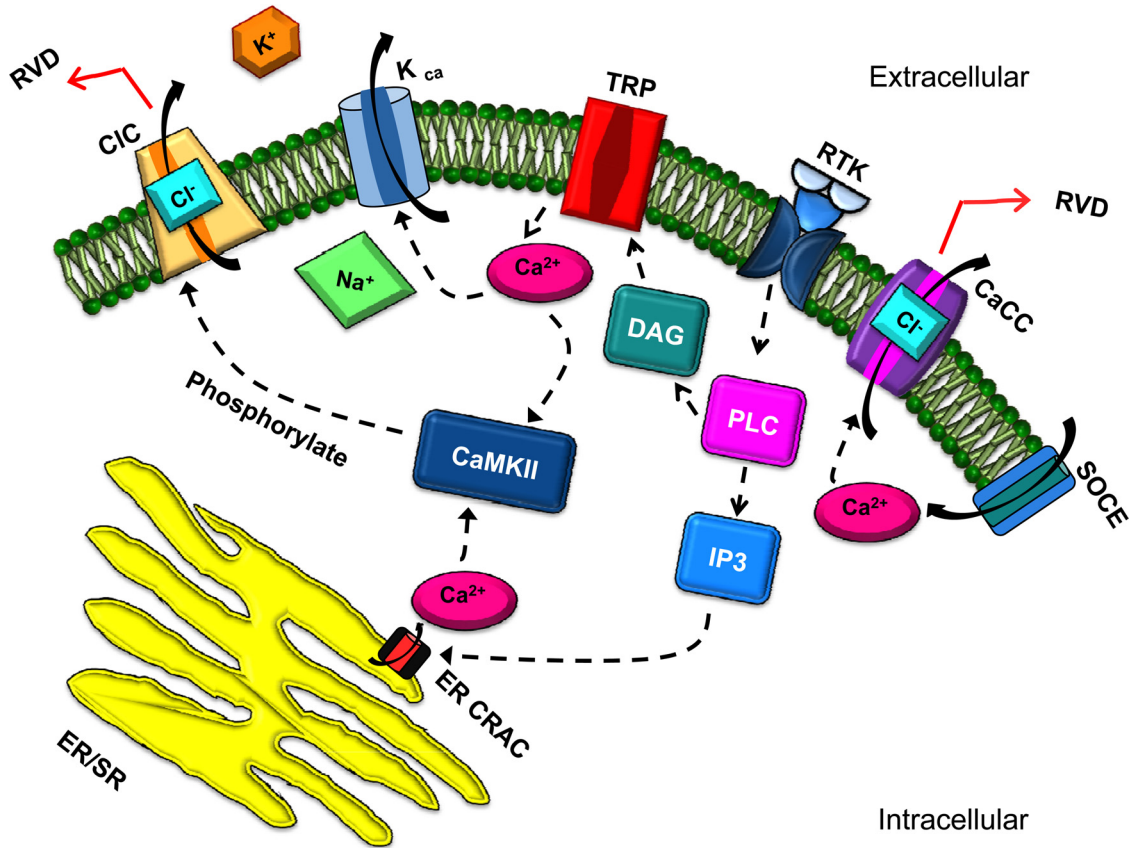

Fig. 1. Increase in cytosolic Ca^{2+} results in activation of Cl^- channels which in turn leads to regulatory volume decrease in glioma. Elevated level of Ca^{2+} in ER activates Ca^{2+} release-activated channels (CRAC) through PLC/IP3 signaling which release Ca^{2+} in to cytoplasm. Activation of SOCE on the plasma membrane as well as TRP channels enhances Ca^{2+} influx. Elevated Ca^{2+} concentration in the cytosol in turn increases Cl^- current via Ca^{2+}-activated Cl^- channel (CaCC) and promotes activation of Ca^{2+}-activated K^+ channel (K_{Ca}) activity. This phenomenon leads to regulatory volume decrease (RVD). Increases in the concentration of cytosolic Ca^{2+} activates Ca^{2+}/calmodulin-dependent protein kinase (CAMKII), leading to phosphorylation of Cl^- channel family (ClC) and thus its activation, specially ClC-3 (which promotes RVD in response to $I_{Cl,swell}$).

Ca^{2+} signalling patterns can be conducted via a series of Ca^{2+} storage discharges, known as Ca^{2+} oscillations [35], which are co-ordinated by store-operated Ca^{2+} entry (SOCE) via Ca^{2+} release-activated calcium (CRAC) channels [31]. It is not known whether Ca^{2+} oscillations are affected by Cl^- channel activity; however, Ca^{2+} oscillations can regulate Cl^- channels such as Ca^{2+}-activated Cl^- channels. Ca^{2+} oscillations initiate the depolymerization of actin which assists tumour proliferation, migration and invasion by promoting a counteracting cell volume increase via activation of Na^+/H^+ exchangers and $K^+/Na^+/Cl^-$ transporters [31]. CRAC is made up of Orai channels and STIM sensors, which respond to Ca^{2+} levels in the ER and SR [24,30]. Ca^{2+} ions are released from the intercellular Ca^{2+} store mainly via inositol 1,4,5-trisphosphate receptor (IP_3R), and ryanodine receptor (RyR), and extruded to the extracellular domain via ATP-driven Ca^{2+} pumps and Ca^{2+}-related exchangers [34]. Glioma exhibits an increase in cytosolic Ca^{2+} through activation of the PLC/IP_3 pathway [32] (Fig. 1).

2.2. Chloride channels

Cl^- channels, the primary anion channels in human cells, are classified based on properties such as voltage-gating, ligand-gating, intracellular phosphorylation, ATP hydrolysis, and cell-volume swelling [36]. They play a vital role in cell volume regulation. In response to hypotonic conditions, swelling-activated Cl^- channels induce ion efflux to reduce swelling by coordinating with other ion channels, pumps, and co-transporters [36]. However, this cell shrinkage assists in glioma invasion throughout the brain [37]. Moreover, Cl^- channel activity is strongly correlated with glioma cell cycle progression [38].

2.2.1. Ca^{2+}-activated Cl^- channels (CaCC)

The molecular identity of CaCC is still controversial. Candidates include TMEM16A, CLCA, ClC-3 and bestrophins. Nonetheless, recent evidence strongly supports TMEM16A as the most likely candidate [39].

2.2.1.1. Ano-1 (TMEM16A). This CaCC channel family is ubiquitously expressed in all tissue types and consists of 10 members. TMEM16A can regulate the activity of other Ca^{2+}-activated channels via linking Ca^{2+} stores from IP_3 and ER to the plasma membrane. In addition, it exhibits an outwardly rectifying, time-dependent current, and is thought to regulate cell volume [10]. Upregulation of TMEM16A has been reported in other cancers to promote cell proliferation through increased activity of the ERK pathway, which is also prominent in glioma.

TMEM16A has been reported to have both Ca^{2+} and voltage-dependence. At low intracellular Ca^{2+} concentration, TMEM16A exhibits an outward Cl^- current when the cell is depolarized. Interestingly, TMEM16A can be activated even at hyperpolarized potentials if Ca^{2+} is present. However, in the absence of Ca^{2+}, the channel is inactivated [40].

2.2.1.2. Bestrophin-1 (Best1). This CaCC family is comprised of four members, and in humans, found in the retina and brain [10]. The current of Best1 exhibits an outward rectification in response to cell volume increase [41]. Best1 is typically localized to the ER and

interacts with the Ca^{2+} sensor, STIM1. When Ca^{2+} is released from the ER, Best1 acts as a counter ion channel and assists in activation of TMEM16A [42]. Retinal pigment epithelium (RPE) cells expressing Best1 mutants had reduced performance in regulated volume decrease [11]. In glial cells, Best1 is responsible for astrocytic GABA and glutamate release. Best1 is also expressed in glioma, and has been speculated to play a role in tumour volume regulation [43].

2.2.1.3. Chloride channel regulator (CLCA). In humans, CLCA exists in three forms: hCLCA1, hCLCA2, and hCLCA3. CLCA1 and CLCA2 can both increase TMEM16A activity [44]. Whereas CLCA1 directly interacts with TMEM16A, CLCA2 interacts with STIM1 and Orai1, and enhanced SOCE upon exhaustion of cytosolic Ca^{2+} [44]. CLCA mediates Cl^- conductance, and can affect cell-cell adhesion, cell cycle progression and apoptosis [45]. Expression of CLCA has been associated with asthma and cystic fibrosis. Moreover, it plays a key anti-cancer role by activating the tumour suppressor p53, and thus can be exploited as a potential therapeutic target for treatment of glioma. However, reports of CLCA expression in the human brain have been elusive [45].

2.2.2. Voltage-gated Cl^- (ClC) superfamily

ClCs consist of 18 segments assembled in a tiled configuration interweaved into the cell membrane [46]. ClCs are divided into three groups: 1) ClC-1, ClC-2, hClC-Ka and hClC-Kb; 2) ClC-3 to ClC-5; and 3) ClC-6 and -7 [47]. The involvement of ClC has been reported in many cancer types [48]. Expression of CLCs in tumours, including glioma, is upregulated, although downregulation of CLC has been reported in other types of cancers [30,42]. Specifically, ClC-1 [49], ClC-2, ClC-3 and ClC-5 [37] are expressed in glioma. Within these, ClC-2 and ClC-3 are overexpressed and enhance glioma cell viability, proliferation, migration and invasion [37]. Knockdown of ClC-3 by shClC-3 adenovirus reduced the nuclear translocation of the p65 subunit of nuclear-factor κB (NF-κB) in glioma and diminished the transcriptional activity of NF-κB. Also, the expression of MMP-3/9, which has been associated with glioma progression, is suppressed when ClC-3 is silenced [50].

In glioma, Cl^- conductance contributes to a large portion of the overall conductance of the cell. Thus, the overall cellular membrane potential lies close to the Cl^- reversal potential [51]. In glioma, applying a hyperpolarizing voltage between −80 mV to −120 mV (relative to a resting potential of −40 mV) induces a voltage- and time-dependent inward Cl^- current [37]. Glioma cells also exhibit a voltage-dependent outward Cl^- current from −60 mV to +100 mV [37]. Inwardly- and outwardly-rectifying currents *(following hyperpolarization and depolarization)* were reduced with knockdown of ClC-2 and ClC-3, respectively. Additionally, the rate at which the ClC-3 outward current increases along the voltage steps can be enhanced by addition of intracellular Ca^{2+} [42], thus indicating that Ca^{2+} plays a role in regulating Cl^- channel conductance. Cl^- conductance in glioma changes cyclically throughout cell cycle progression, where it is highest in the early G_1 phase and lowest in the S phase [52]. Ca^{2+} has also been observed to change during the cell cycle [53], thereby indicating a possible relationship between Ca^{2+} and Cl^- currents in glioma cell cycle progression.

Early detection of glioma is difficult due to their invasion into the brain's white matter, and not apparent until patients exhibit pain or impaired motor activity [54]. Early signs of glioma include brain swelling, which causes intracellular pH deregulation, and changes in signalling substrates and enzyme concentration [55]. Cl^- channels can induce decrease in cell volume by initiating passive Cl^- efflux, which is accompanied by increase in K^+ conductance [17]. This results in surrounding aquaporins to efflux water which leads to RVD [51]. In order to maintain constant Cl^- and salt efflux, Cl^- transporters are required to actively bring Cl^- back into the cell to maintain both the membrane potential and the electrochemical gradient [51]. RVD has been shown to assist glioma invasion throughout the tight brain parenchyma by changing cell volume and shape [17] (Fig. 2).

2.2.2.1. Swelling-induced Cl^- currents ($I_{Cl,swell}$). An increase in anionic membrane permeability following cell-swelling was first reported ˜40 years ago [56]. It was later shown that this increase in permeability caused $I_{Cl,swell}$, which facilitated subsequent regulatory volume decrease (RVD) via volume regulated anion channels (VRAC) [57]. However, volume sensitive outward rectifying (VSOR) channels and volume sensitive organic osmolyte/anion channels (VSOAC) have also been attributed to RVD [10]. It is unclear whether VRAC, VSOR, and VSOAC are composed of related proteins or separate distinct entities, and the identity of the Cl^- channel(s) responsible for $I_{Cl,swell}$ remains ill-defined. One of the early candidates for VRAC was the leucine-rich repeat containing 8A (LRRC8A), which is a widely expressed Cl^- channel regulated by cell volume and Ca^{2+} [58]. The action of mechanism of LRRC8A in volume regulation is unknown, but it is strongly suggested that LRRC8A must form a heterodimer with an additional LRRC8 protein to form functional VRAC [51]. Impairment of LRRC8A alone was detrimental to cell volume regulation. Specifically, suppression of LRRC8A in HEK cell lines, HeLa and T-lymphocyte cell lines prevented RVD through inactivation of $I_{Cl,swell}$ [58]. Moreover, glioma was unable to perform RVD after silencing of LRRC8A, which resulted in reduced cell viability and proliferation, as well as increased sensitivity to chemotherapeutic drugs temozolomide and carmustine [59].

Importantly, certain members of the ClC family can also be activated by cell swelling to generate $I_{Cl,swell}$, notably ClC-2 and ClC-3 [60,61]. However, there is controversy in the literature whether ClC-2 or ClC-3 is the predominant channel in generating $I_{Cl,swell}$, with multiple studies supporting and refuting their importance. ClC-2 expressed in *Xenopus* oocytes activated in response to hypotonic solution-induced cell-swelling [62]. In SF9 cells, CLC-2 promoted RVD [63]. Consistent with this, knockdown of ClC-2 decreased volume regulation in hepatoma cells [64]. However, ClC-2 knockout mice did not experience a change in RVD compared to control [65], and ClC-2 current in T_{84} cells was only modulated by cell-swelling when exposed to hyperpolarization [51]. For ClC-3, early studies demonstrated that its downregulation suppresses RVD in HeLa cells [61] and inhibits $I_{Cl,swell}$ in rat brain epithelial cells [66]. However, overexpression of ClC-3 in HEK293 cells did not affect $I_{Cl,swell}$ and had no role in RVD [67]. Furthermore, in human pulmonary artery smooth muscle cells, ClC-3 knockdown did not affect $I_{Cl,swell}$ [68]. Additionally, $I_{Cl,swell}$ was not affected in ClC-3 knockout mice [69]. These contrasting results suggest that whether CLC-2 or CLC-3 is the dominant player in generating $I_{Cl,swell}$ may be cell type dependent.

Nonetheless, the most recent and strongest candidate for $I_{Cl,swell}$ in glioma is ClC-3. It was reported that ClC-3 coordinates with other Cl^- channels and co-transporters to maintain cell volume, and inhibition of ClC-3 alone is insufficient to mitigate glioma invasion [48]. ClC-3 is a membrane delimited channel that is activated by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation [70] (Fig. 1). Expression of ClC-3 is 10 folds higher in glioma than in non-malignant brain tumours [70]. ClC-3 activation by CaMKII indicates that changes in intracellular Ca^{2+} concentrations can play a role in conducting $I_{Cl,swell}$, further suggesting synergy between Ca^{2+} and Cl^- currents in promoting glioma malignancy.

2.2.2.2. Effects of $I_{Cl,swell}$ on glioma signalling and cellular functions. Activity of ion channels is regulated by various signalling proteins and their associated pathways [12]. Signalling pathways that act via phosphorylation of tyrosine, threonine, and serine residues regulate most voltage gated ion channels, including those in the ClC family, by altering their voltage sensitivity [30] or by promoting flux of signalling molecules between organelles within the cell [71]. Ion channels also associate with cell-adhesion molecules such as integrin, which create cell signalling complexes with various other receptors. Activity of these cell-adhesion molecules can affect cell differentiation and neuron outgrowth [12].

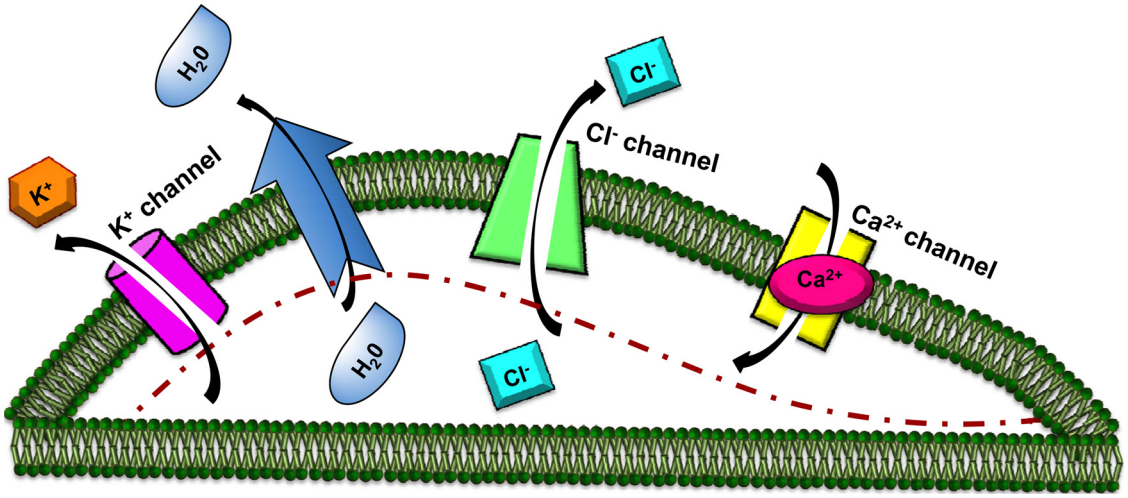

Fig. 2. Role of Cl^-, Ca^{2+}, and K^+ channels in regulatory volume decrease (RVD) in glioma. In response to cell swelling, upregulated Ca^{2+} channel activity *(from either surface membrane or ER)* increases intracellular Ca^{2+} which in turn enhances K^+ and Cl^- channel activity. Increased effluxes of Cl^- and K^+, along with net efflux of water, lead to induction of glioma cell shrinkage and RVD. Cell shrinkage plays a major role with glioma migration and invasion.

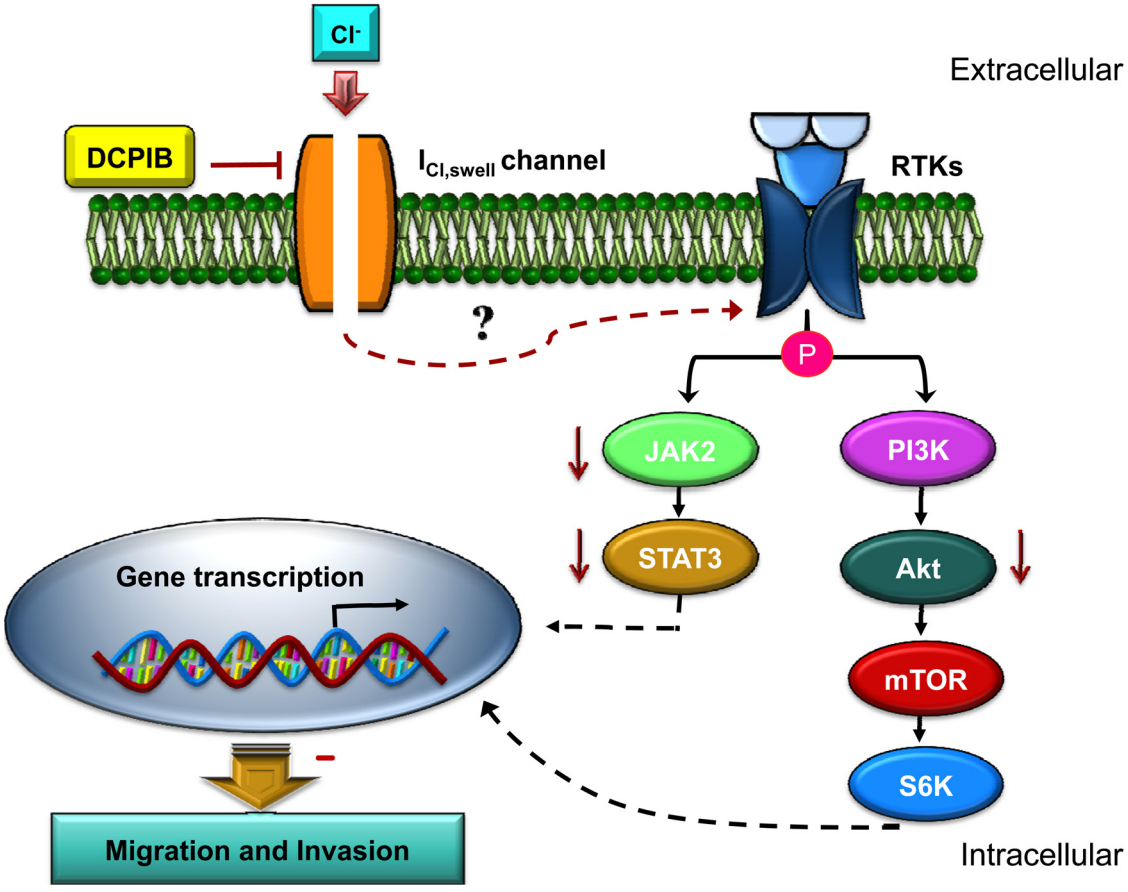

Fig. 3. Inhibition of swelling-induced Cl^- current ($I_{Cl,swell}$) by DCPIB decreased glioma migration and invasion. Cl^- flux is regulated through swelling induced Cl^- channels in response to ionic homeostasis. The suppression of the $I_{Cl,swell}$ potentially affects RTK activity which mediates the JAK2/STAT3 and PI3K/Akt pathways. Downregulation of these pathways results in alteration of gene transcription and translation involved in glioma migration and invasion. Inhibition of $I_{Cl,swell}$ by DCPIB lead to reduced Akt and JAK2/STAT3 signaling, which can underlie the observed suppression of proliferation, invasion and migration in GBM.

We recently investigated the pharmacological effects of DCPIB, a selective inhibitor of $I_{Cl,swell}$, on U87 and U251 GBM cell viability, proliferation, migration, and invasion. We found that glioma exhibited $I_{Cl,swell}$ following hypotonic solution-induced cell swelling, and this current was abolished with application of DCPIB. Moreover, inhibiting $I_{Cl,swell}$ suppressed glioma cellular functions, 100 μM DCPIB treatment of U87 and U251 cells reduced cell viability and colony formation at 48 h. Glioma migration and invasion were also significantly inhibited by DCPIB [12]. It is speculated that a higher *in vitro* concentration of DCPIB was required due its chemical instability over two days in culture. In GBM, the JAK/STAT3 and PI3K/ATK pathways are hyperactivated and found to be essential for cell viability and migration [72].

We observed that DCPIB reduced JAK2/STAT3 and Akt/PI3K signaling, and thus speculate that these pathways are involved in the underlying mechanism by which $I_{Cl,swell}$ suppression inhibits GBM cellular functions [16]. Specifically, we propose that $I_{Cl,swell}$ could activate these pathways via phosphorylation by receptor threonine kinases (RTKs) (Fig. 3).

Our findings are consistent to those reported by Sforna et al. [73], whose group demonstrated in GBM the mechanism by which Cl^- channels induced $I_{Cl,swell}$ in response to acute and chronic hypoxia. Similarly, they observed that $I_{Cl,swell}$ generated by cell-swelling resulted in RVD. This was hypothesized to prevent cell necrosis following hypoxic stress. However, following chronic hypoxia, GBM cells had suppressed $I_{Cl,swell}$ and slower RVD. In the GL-15 human GBM cell line, Catacuzzeno et al. [17] reported that $I_{Cl,swell}$ required the activity of U73122-sensitive phospholipase C (PLC), membrane permeable diacylglycerol (DAG), and EHT1864-sensitive Rac1 small GTPase [17]. They also found that $I_{Cl,swell}$ can be activated by fetal calf serum (FCS), which suggested that leakage of the blood brain barrier potentially plays a role in tumour cell invasiveness [17].

2.2.3. Other types of Cl^- channels

2.2.3.1. Cystic fibrosis transmembrane conductance regulator (CFTR). Consisting of 12 membrane spanning segments and two nucleotide binding domains (NBD1 and 2) plus a regulatory R domain, CFTR is activated via cAMP-dependent phosphorylation and binding of ATP at the nucleotide binding domains [46]. CFTR acts as a conductance regulator of several channels such as suppression of CaCC and the outwardly rectifying Cl^- channel (ORCC) [74]. It also acts as a regulator of TRPV4, which was shown to deliver the Ca^{2+} signal for RVD in epithelia [10]. CFTR modulates inflammation and apoptotic pathways, and a CFTR gene mutation has been associated with cervical and pancreatic cancer [48]. Thus, CFTR can potentially also play a role in glioma, although its role here has yet to be reported.

2.2.3.2. Cl^- intracellular channel protein (CLIC). Also known as the p64 family, CLIC consists of six members: CLIC1, CLIC2, CLIC3, CLIC4, CLIC5, and CLIC6 [9]. Their main roles include regulation of endosomal trafficking, actin-dependent membrane remodelling, and tubulogenesis [9]. Additionally, both overexpression and underexpression of CLIC1 and CLIC4 have been observed in several tumour cell lines and have been considered to be therapeutic targets for chemotherapy [48]. CLIC4 is regulated by p53 and tumor necrosis factor α (TNFα), and has been observed to translocate from the plasma membrane to the nucleus in response to stressful conditions to assist in apoptosis, thus highlighting its role in tumour suppression [48]. Specifically, in glioma, suppression of CLIC4 has been reported to increase apoptosis induced by hydrogen peroxide [50]. Furthermore, CLIC1 expression is upregulated in GBM at higher levels than low grade glioma [48]. Suppression of CLIC1 in GBM decreases cell proliferation and hinders the self-renewal ability of cancer stem cells [75].

2.2.3.3. Maxi Cl^- channel. Found in multiple cell types (including glia, neurons, lymphocytes, macula densa, and cardiac muscle cells), maxi Cl^- channels are involved in controlling cell membrane potential, apoptotic pathway and cell volume regulation. Swelling, hypoxia and ischemia activate this channel [76], which suggests its potential role in glioma cellular functions.

2.2.3.4. Glycine-gated Cl^- and GABA channels. These heteropentameric proteins are comprised of three subunits (α, β and γ), which in turn constitute four different membrane-integrated segments, building up the core side of the channel from the C-terminal side and are extended to the extracellular surface through the N-terminal side. This creates the ligand binding site of the channel [46]. $GABA_A$ is a Cl^- channel comprised of eight subfamilies, which differ in functionality and pharmacologically based on their subunit structure [3]. Physiologically, $GABA_A$ receptor channels enable Cl^- flux into neurons to counteract depolarization and suppress action potentials [3]. Nevertheless, GABA signalling is also involved in growth regulation of neuronal stem cells, neuroblasts and neuronal tumour cells. In glioma, the decrease in GABA receptors has been correlated with the tumour's degree of malignancy. GABA receptors are absent or misfolded in GBM which could attribute to its enhanced malignancy [3].

2.2.4. Role of Cl^- channels in glioma cell proliferation, apoptosis, migration and invasion

2.2.4.1. Proliferation. When the cell experiences adverse conditions, CLIC-1 activity enables Cl^- influx during the G1/S phase of the cell cycle to induce proliferation [75,76]. Inhibition of CLIC-1 can prolong the duration of the cell cycle [79]. GBM cell lines have been shown to overexpress CLIC-1 proteins in order to promote proliferation [80]. Pharmacological inhibition or knockdown of CLIC-1 decreases GBM proliferation and tumorigenesis [30]. In GBM derived cancer stem cells (CSC), CLIC-1 is secreted in extracellular vesicles (EV). Administration of CLIC-1 containing EVs promotes cell proliferation in GBM cells, while lowered levels of CLIC-1 in GBM EVs decreased cell growth [80].

For mitosis to be successful, cell volume decrease for pre-mitotic condensation (PMC) is required prior to the M phase [30]. During PMC, ClC-3 membrane expression is elevated. Suppressing ClC-3 halts PMC and thus cell cycle progression. Furthermore, inhibiting GABA-gated Cl^- channels in the U3013 GBM cell line stunted cell growth. This strongly suggests that Cl^- current is required for cell proliferation and differentiation [5].

2.2.4.2. Apoptosis. Glioma requires Cl^- channel activity to initiate an apoptotic volume decrease (AVD). Inhibition of Cl^- channels prevents AVD in addition to caspase activation and DNA fragmentation [30]. Knockdown of CLIC4 in glioma results in an elevation of apoptosis induced by hydrogen peroxide. Although Bax is associated with CLIC4 in apoptosis, Bax/Bcl2 expression did not change even with CLIC4 suppression [81]. However, when glioma was exposed to hypoxia, CLIC4 was upregulated along with an increase in Bax/Blc2 and caspase-3 expression [81]. However, Bax-induced apoptosis was not prevented by CLIC4 suppression, which suggested different signalling pathways [78]. CLIC4 is also involved in p53 and cMyc activated apoptosis [48]. When U251 cells were starved, CLIC4 inhibition and subsequent upregulation of beclin 1 resulted in enhanced autophagy. CLIC4 inhibition also induced mitochondrial and ER apoptosis as a result of Bax/Bcl-2 and cytochrome c release, which increased caspase e and C/EBP homologous protein (CHOP), respectively [82]. In C6 cells, silencing the ClC-4 gene resulted in hydrogen-induced and TNFα-mediated apoptosis [81].

2.2.4.3. Migration and Invasion. To traverse the narrow parenchyma space within the brain, glioma undergoes cell volume decrease, which is primarily regulated by Cl^- flux. Inhibition of Cl^-channels reduces glioma migration [30]. When treated with the Cl^- inhibitor NPPB, glioma crossed the Transwell membrane at a slower rate [28]. Replacing Cl^- with I- or Br^- allowed glioma to retain its original ability to migrate [28].

Invasion of glioma is mediated by Cl^- and K^+ efflux, which results in RVD [28]. GABA-gated Cl^- channels can induce Cl^- accumulation and thus regulate RVD [28]. Na-K-Cl cotransporter (NKCC) is the main transporter in glioma responsible for Cl^- accumulation. K^+, Na^+, and two Cl^- ions are transported by NKCC into the cell using the downward Na^+ gradient. Thus, the increase in Cl^- gradient can ultimately induce cell volume changes to facilitate migration and invasion in glioma [83]. The uptake of Cl^- is balanced by efflux of Cl^- via KCC1 and KCC3a transporters, which can regulate migration [28]. Swelling-induced Cl^- currents have also be shown to contribute to migration and invasion in glioma via activation of JAK2/STAT3 and Akt signaling pathways (Fig. 3) [16]. ClC-3 forms protein complexes with matrix

Table 1
Summary of the mechanistic effects of pharmacological channel inhibitors on GBM cellular functions.

Name of compound	Mechanism	Effects on GBM Cellular Functions	Ref
DIDS	VSOR inhibitor, RVD inhibitor, anti-apoptotic, blocks hypoxia-induced swelling	Reduced cell viability and proliferation	[38,55,69,89]
NPPB	CLC3, ClC1, CFTR, inhibitor, blocks hypoxia-induced swelling, RVD inhibitor	Decreased cell invasion	[55,69,90]
DIOA	K-Cl cotransporter inhibitor, RVD inhibitor	Decreased cell invasion	[55]
DCPIB	ICL swell inhibitor, RVD inhibitor	Reduced cell viability and proliferation Suppressed cell migration and invasion	[16,69,94]
CTX	CLC3 inhibitor, RVD inhibitor	Suppressed cell migration and invasion	[12,29,87,95]
Metformin, phenformin, moroxydine	CLIC1 inhibitor	Suppressed cell proliferation	[77,96]
Ani9	TMEM16 inhibitor	Suppressed cell proliferation	[97]
TEA	Potent VRAC inhibitor, weak RVD inhibitor	Suppressed cell migration and invasion	[95]
Tamoxifen	Potent VRAC inhibitor, RVD inhibitor	Suppressed cell migration and invasion	[95,98]

metalloproteinase-2 (MMP-2), and has been observed to co-localize with K_{Ca}channels in the lipid raft domain of invadipodia [78].

3. Interaction between calcium and chloride

3.1. Effects of Ca^{2+} on Cl^- signalling

Ca^{2+} can influence glioma migration and invasion via modulation of Cl^- channel activity [42]. Elevated Ca^{2+} influx through TRP channel, TRPC1, is essential for epidermal growth factor (EGF)-induced invasion in several glioma cell lines [12]. Furthermore, this Ca^{2+} influx also activates CAMKII-activated ClC-3 activity, which results in an efflux of Cl^- ions and RVD for cell migration (Fig. 1). Similarly, in GBM cell lines, potentiated K_{Ca}3.1 and ClC-3 activity by bradykinin was necessary for chemotactic cell migration [14]. Bradykinin was found to interact with GPCR receptors which led to increased intracellular Ca^{2+}. This resulted in upregulated K_{Ca}3.1 as well as CAMKII-activated ClC-3 activity, causing Cl^- and K^+ efflux that resulted in cell volume decrease required for GBM migration and invasion [14]. In U87 cells, FCS was shown to increase activity of K_{Ca}3.1through Ca^{2+} oscillations, and subsequently increased Cl^- channel activity. This decreased cell volume and assisted glioma migration [15].

Several CaCC channel genes are upregulated, including anoctamin, Cl^- channel regulator (ClCA) and bestrophin [42]. Moreover, TMEM16A is found to be overexpressed in glioma and helps to promote cell proliferation, migration and invasion [84]. The proposed underlying mechanism is that upregulated TMEM16A activates the NF-κB signalling pathway which increases expression of oncogenes (e.g. cyclin D1, cyclin E, and cmyc). The NF-κB pathway also promotes activity of MMP-2 and MMP-9, which regulate the structural integrity of the ECM in the brain and promote glioma migration and invasion [84]. GABA-gated Cl^- channels also increase intracellular Ca^{2+} concentration in glial cells, since hyperpolarization from Cl^- influx induces activation of voltage-gated Ca^{2+} channels, resulting in Ca^{2+} influx [22].

3.2. Effects of Cl^- on Ca^{2+} signalling

Inversely, Cl^- channels can also regulate cellular Ca^{2+} and its associated signalling pathways. $GABA_A$ receptors mediate Cl^- currents, which can hyperpolarize the membrane potential resulting inhibition of cell firing in neurons. However, in astrocytoma and oligodendroglioma, $GABA_A$ receptor channels can also depolarize the cell while intracellular Cl^- concentrations are high. Depolarization causes an increase in intracellular Ca^{2+} via the activation of voltage activated Ca^{2+} channels (VGCC) [82,18]. Elevation of intracellular Ca^{2+} by VGCCs led to uncontrolled proliferation and migration in cancer [18]. In neurons, activation of CaCC channels following Ca^{2+} influx caused an efflux of Cl^-, which depolarized the cell and activated VGCCs for even more Ca^{2+} influx and thus further depolarization [86]. Although this continuous depolarization has not been reported in glioma, inhibition of VGCC to reduce Ca^{2+} entry decreased glioma cell proliferation and induced apoptosis [18]. This suggests that intracellular Ca^{2+} elevation induced by hyperpolarization is important for the activation of Ca^{2+}-dependent proliferative and survival pathways.

4. Pharmacology

There is growing evidence of the involvement of Cl^- channels in cancer pathology, illustrating their role as potential therapeutic targets for chemotherapy [36,8]. Volume-activated Cl^- currents can be inhibited by an array of compounds, which are divided into the following classes: Class I compounds are transported into the cell via phosphoglycoprotein (P-gp) and stop channel activation without blocking the pore. These compounds react inside the cell following ATP hydrolysis; Class II compounds are transported into the cells vial P-gp and physically block the channel by binding to its extracellular area. Note that some class II compounds (e.g. tamoxifen) are P-gp independent and bind to other receptors; and Class III compounds (e.g. DIDS and NPPB) are unable to suppress P-gp- dependent drug transport, but can block volume-activated Cl- current [84,85]. Refer to Table 1 for summary.

DIDS, a classical VSOR and RVD inhibitor [55] (Fig. 4), acts as an anti-apoptotic compound by increasing the cell's water content. Additionally, in epidermoid cancer cells, DIDS prevented decrease in cell viability when trichostatin (TSA) and cisplatin were also added simultaneously [89]. Treatment of TSA and cisplatin increased caspase-3 activity, which induced apoptosis and decreased cell viability [89]. Hypoxia-induced swelling was blocked by DIDS (30 mmol/l) and NPPB [69]. In astrocytoma, DIDS, DNS and Zn^{2+} decreased cell proliferation [38].

Additionally, RVD is also inhibited by NPPB and Cd^{2+} [55]. Treatment of glioma with these inhibitors combined resulted in greater inhibition of RVD than when used separately [55]. NPPB was reported to completely suppress glioma cell invasion [90] (Fig. 4). However, GBM cells were potentially overdosed and cell invasion was halted due to cytotoxic levels of NPPB [91]. Note that NPPB is non-specific and can inhibit the function of multiple Cl^- channels (e.g. ClC-2, ClC-3, and CFTR) [37], as well as K_{Ca} channels [89,88].

The alkylating agent temozolomide (TMZ) is the current standard chemotherapeutic drug for GBM treatment [92]. TMZ can conjugate with NPPB, creating TMZ-NPPB, which blocks Cl^- currents (with similar efficacy as NPPB alone) in glioma, and ultimately suppresses cell viability, migration and invasion [13]. TMZ-NPPB is more stable than TMZ, thus having greater therapeutic potential for treating glioma [13]. Furthermore, sensitivity to TMZ can be restored by inhibiting PI3K [93].

DCPIB is a selective $I_{Cl,swell}$ inhibitor which can also impede RVD [68]. After DCPIB treatment, we observed reduction of intracellular Cl^- in PC12 cells [94]. In addition, DCPIB abolished $I_{Cl,swell}$ in GBM, and suppressed glioma viability, proliferation, colony formation, migration and invasion [16].

Chlorotoxin (Ctx), an inhibitor of small conductance Cl^- channels (Fig. 4), is currently under phase I/II clinical trials for glioma treatment

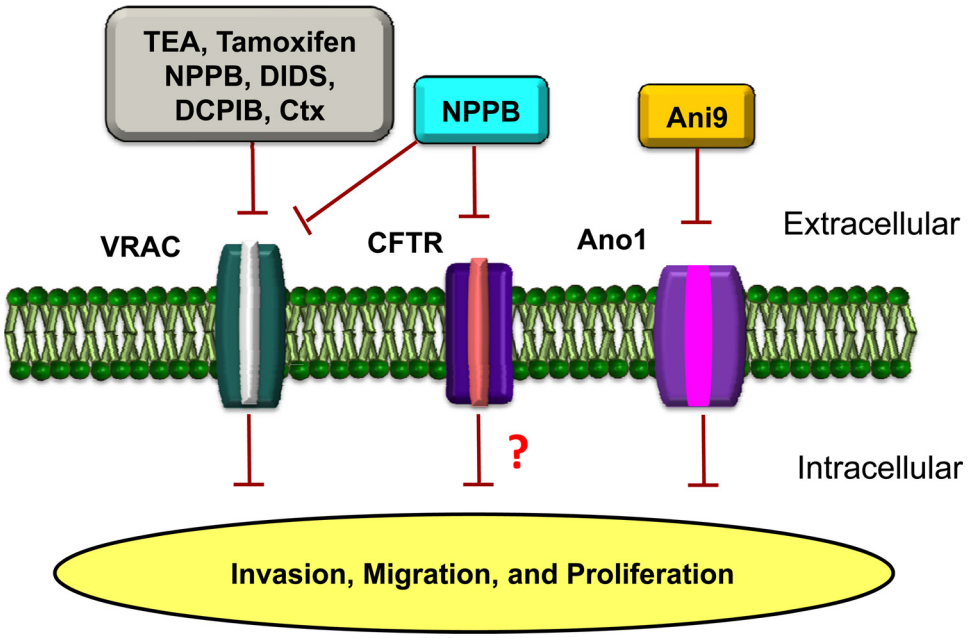

Fig. 4. Schematic representation of potential pharmacological inhibitors for glioma treatment. Non-selective Cl^- channel inhibitors DIDS, DCPIB, and Ctx inhibit VRAC, while NPPB blocks both VRAC and CFTR channels. DIDS and NPPB can block hypoxia-induced swelling. DCPIB impedes $I_{Cl,swell}$and down-regulates JAK2/STAT3 and Akt signaling pathways, which results in suppression of proliferation, invasion and migration. Tamoxifen and K^+-channel inhibitor TEA suppress Cl^- currents conducted through VRAC. This ultimately decreases RVD in glioma, and thus suppresses migration and invasion. The small molecule inhibitor Ani9 selectively inhibits Ano1. Suppression of VRAC, Ano1 (and potentially CFTR) leads to decreased invasion, migration, proliferation in glioma.

[48]. Action of mechanism of Ctx is via internalization of a complex formed by MMPs and ClC-3 at the cell membrane [30]. Ctx selectively inhibits ClC-3 expression [12], and suppresses glioma migration both *in vitro* cell cultures and *in vivo* mouse models [29,87]. Knockdown of ClC-3 with siRNA in addition to Ctx treatment only had slight increase in efficacy compared to Ctx alone, suggesting that Ctx is a potent ClC-3 inhibitor [78]. Ctx (5 μM) prevented glioma invasion into fetal rat brain aggregates and was able to irreversibly block RVD [95]. The Ctx derivative, TM-601, has been used in clinical trials to treat high grade glioma [30]. Ctx derivatives, CA4 and CTX-23, were designed from sequences of Ctx and BmKCT, a scorpion Cl^- toxin. CA4 and CTX-23 inhibited glioma cell growth and migration as well as decreased cell extensions and increased the diameter of nuclei in glioma cells. Cytotoxicity of Ctx and its derivatives was specific to glioma, and there was insignificant reduction in cell viability of healthy neurons and astrocytes [80].

Metformin, traditionally used as a drug for type-2 diabetes, has also been reported to have anti-tumorigenic properties. Specifically, metformin suppresses cell proliferation in GBM cell lines at an EC_{50} of 23, 6.6 and 1.7 mM (for 24, 48 and 72 h, respectively). Metformin acts by directly binding to ClIC-1 and preventing Cl^- conductance,which consequently suppresses cell proliferation [96]. Related biguanides such as phenformin, moroxydine, and cycloguanil show similar mechanisms of ClIC-1 inhibition, and also suppress cell proliferation to varying efficacy [77].

Ani9 is a selective inhibitor of TMEM16A. In glioma, Ani9 (IC_{50} < 3 μM) potently suppressed TMEM16A activity. Due to its high specificity, Ani9′s suppression of overall VRAC function was lower than that of less selective CaCC channel inhibitors such as $T16A_{inh}$-A01 and NPPB. Additionally, increasing the concentration of Ani9 did not result in greater inhibition of VRAC [97] (Fig. 4).

Finally, tamoxifen (10 μM), a commonly used medication for breast cancer prevention [98], and the K^+ channel blocker TEA (1 mM), have been reported to reduce Cl^- flux resulting in a decrease in glioma cell invasion and migration [95]. We speculate that a high TEA concentration was necessary to elicit *in vitro* effects due to the indirect mechanisms of inhibiting Cl^- current via K^+ channel activity suppression. These compounds also reduced RVD in glioma cells that were subjected to osmotic swelling [95] (Fig. 4), suggesting that they may be potent inhibitors of VRAC.

4.1. Multidrug resistance (MDR)

Tumour cell resilience to chemotherapy is a major challenge in glioma treatment. MDR is characterized as resistance to an array of anti-cancer drugs with differing structure and mechanism of action [99]. MDRs expel anti-cancer drugs by ATP- dependent proteins within the ATP-binding cassette (ABC) transporter superfamily, which includes P-gp, multidrug resistance protein (MRP), and major vault protein (MVP) [96,97].

P-gp plays important roles in translocating substrate molecule. Malignant tumours express P-gp in order to protect themselves during chemotherapy. Increase in P-gp expression before and after therapy indicated that P-gp is potentially involved in intrinsic and acquired MDR in glioma. Increase in Cl^- current in response to cell swelling has been correlated with upregulation of P-gp [101,102]. It was initially debated whether P-gp was a Cl^- channel itself, or a Cl^- channel regulator. It was later demonstrated that suppression of P-gp did not abolish swelling-induced currents, thus suggesting that P-gp does not have intrinsic Cl^- channel properties [103].

GBM showed higher MDR expression than low grade astrocytoma [100]. Overexpression of MRP1 has been associated with MDR [100]. Expression of MRP1 is higher in Grade III and IV glioma compared to lower grade glioma [104]. Increase in MRP1 expression is suggested to correlate with intrinsic or extrinsic drug resistant [105,106]. Overexpression of MVP is also seen in many tumours including glioma [96,102]. Increase in MVP expression is observed after chemotherapy [100].

4.2. Therapeutic potential for glioma

Combination drug therapy can improve glioma treatment outcomes. Uptake of doxorubicin in U87 cells was increased upon treatment with antisense oligodeoxynucleotides, a P-gp inhibitor [107]. The MRP inhibitor indomethacin enhanced the cytotoxic effects of etoposide and vincristine on multiple glioma cell lines [108]. Silencing MRP1 allowed etoposide to recover its cytotoxic effects against etoposide-resistant T98G-VP and Gli36-VP cell lines [100]. siRNA can also be used to silence MDR1 to induce tumour apoptosis [108].

5. Conclusion

Cl^- channels play a significant role in glioma pathology. The regulation of Cl^- flux is essential for glioma proliferation, migration and invasion. When expressed aberrantly, Cl^- channels can suppress glioma apoptosis and improve viability.

When considering the treatment of glioma, there are two major categories of Cl^- channels that need further examination: 1) those that play a role in generating the swelling-induced Cl^- current, $I_{Cl,swell,}$ and, 2) those that directly affect cell proliferation and growth. $I_{Cl,swell}$ plays a key role in the regulation of cell volume, specifically regulatory volume decrease (RVD), which sustains cell viability, promotes invasion and migration, and initiates proliferation signalling pathways. $I_{Cl,swell}$ is strongly speculated to be generated by ClC-3 in glioma, which represents a potential therapeutic target. Additional potential drug targets of interest include CLIC1 and TMEM16A, which are Cl^- channels that can directly impact glioma cell proliferation and growth.

Furthermore, there is strong evidence in the literature supporting the important relationship between Cl^- and Ca^{2+} signalling in contributing to tumour malignancy. Therapeutic drugs targeting Cl^- channels can exploit this Cl^- and Ca^{2+} interplay, and potentially be pivotal for glioma treatment.

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