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IDENTIFICATION OF COMPOUNDS THAT TARGET GLIOMA INITIATING CELLS

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Identification Of Compounds That Target Glioma Initiating Cells

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To my family

ABSTRACT

Glioblastoma multiforme is a common form of brain tumor that leads to debilitating effects despite the current regiment of treatment, which includes surgery, chemotherapy and radiotherapy. With the discovery of glioma-initiating cells (GICs) that exists within the bulk tumor, some light has been shed on this disease. GICs have been shown to be resistant to chemotherapy and radiotherapy, therefore providing a plausible explanation for the high propensity for recurrence in patients. However, effective treatments are still unavailable, and there is an urgency to discover drugs that can eradicate this group of cells. The overall aim of this thesis is to identify drugs from small molecule screens that can kill GICs, as well as to understand the possible causes and mechanisms for drug sensitivity.

In Paper I, we have identified a new small molecule, Vacquinol-1, that reduced the viability and growth of GICs, both *in vitro* and *in vivo*, and had little effects on normal cells such as fibroblasts and embryonic stem cells. Cell death was caused by an unconventional non-apoptotic manner, where the cells showed massive accumulation of vacuoles that formed through macropinocytosis. This led to the impairment of cell function followed by cell death. Furthermore, treatment with Vacquinol-1 also exhibited excellent improvement in survival of glioma xenografted mice. Using an shRNA screen, mitogen-activated protein kinase kinase 4 (MKK4), which is involved in stress response, was determined to play a role in the unique type of cell death.

In Paper II, we have identified that GICs are particularly sensitive to perturbations in calcium (Ca^{2+}) homeostasis, with the relative degree of sensitivity being linked to their degree of stemness. The two compounds employed in this study, Ca^{2+} ionophore A23187 and the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump inhibitor Thapsigargin, are well-known compounds that elicit cell death. However, the compounds affected viability to a different degree in the various GIC lines, with the line that is more similar to neural stem cells being more sensitive to perturbations in Ca^{2+} homeostasis. This sensitivity was correlated to expression levels of different Ca^{2+} related proteins such as GRIA1 and S100A6, as well as to Nestin (NES).

In Paper III, we have repositioned Niguldipine, an old anti-hypertensive drug, as a potential compound for targeting GICs. Through an ion channel drug screen, we have also observed that GICs exhibit sensitivity to Ca^{2+} modulators, suggesting once again that Ca^{2+} homeostasis is critical to cell viability in GICs. Niguldipine, which is also a Ca^{2+} channel inhibitor, also showed a selection for GICs as compared to normal cells such as fibroblasts and neural stem cells. At the effective dose, the compound showed no effects on cardiac rhythmicity, and administration of the drug resulted in a significant improvement in the survival of glioma xenografted mice.

In conclusion, we have identified a few small molecules, both old and new, that can reduce the proliferation and viability of GICs without affecting that of normal cells. One of the mechanisms underlying selectivity is that GICs show a greater degree of sensitivity to

disturbances in Ca^{2+} homeostasis, therefore suggesting that Ca^{2+} modulators should be screened for their potential in cancer therapy.

LIST OF SCIENTIFIC PAPERS

I. Kitambi, S. S., Toledo, E. M., Usoskin, D., Wee, S., Harisankar, A., Svensson, R., Sigmundsson, K., Kalderén, C., Niklasson, M., Kundu, S., Aranda, S., Westermark, B., Uhrbom, L., Andäng, M., Damberg, P., Nelander, S., Arenas, E., Artursson, P., Walfridsson, J., Forsberg Nilsson, K., Hammarström, L. G. J. and Ernfors, P. (2014), **Vulnerability of Glioblastoma Cells to Catastrophic Vacuolization and Death Induced by a Small Molecule.** Cell, 157: 313-328.

II. Wee, S.*, Niklasson, M*, Marinescu, V. D., Segerman, A., Schmidt, L., Hermansson, A., Dirks, P., Forsberg-Nilsson, K., Westermark, B., Uhrbom, L., Linnarsson, S., Nelander, S. and Andäng, M. (2014) **Selective calcium sensitivity in immature glioma cancer stem cells.** PLoS ONE,

III. Niklasson, M.*, Wee, S.*, Mutlu, E., Kyriatzis, G., Schmidt, L., Nilsson, E., Segerman, A., Forsberg-Nilsson, K., Westermark, B., Linnarsson, S., Uhrbom, L., Vincent, T., Uhlén, P., Nelander, S., Enger, P. Ø. and Andäng, M. **Calcium modulator targets glioma tumor initiating cells.** *Manuscript.*

* Denotes equal contribution

CONTENTS

LIST OF ABBREVIATIONS

ATG8/LC3	Autophagy-related protein 8/Microtubule-associated protein 1 light chain 3
ATP	Adenosine triphosphate
BCL2	B-cell lymphoma 2
bFGF/FGF	Basic fibroblast growth factor
BK	Big potassium channel
BLBP/FABP7	Brain lipid-binding protein/Fatty acid binding protein 7
BMPs	Bone morphogenetic proteins
Ca^{2+}	Calcium
CACNA1	Calcium channel, voltage-dependent, alpha 1 family
CD133	Prominin-1
DCX	Doublecortin
DNA	Deoxyribonucleic acid
EAATs	Excitatory amino-acid transporters
EGF	Epidermal growth factor
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GFAP	Glial fibrillary acidic protein
GICs	Glioma initiating cells
GRIA1	Glutamate receptor 1, Ionotropic, AMPA
hERG	Human Ether-à-go-go-Related Gene
HSPA5	Heat-shock 70kDa protein 5
$Ins(1,4,5)P_3$	Inositol-1,4,5-triphosphate
$InsP_3R$	Inositol-1,4,5-triphosphate receptor
K^+	Potassium
LAMP1	Lysosomal-associated membrane protein
MAP2	Microtubule-associated protein 2
mESCs	Mouse embryonic stem cells
MKK4	Mitogen-activated protein kinase kinase 4
Na^+	Sodium

NCX	Sodium-calcium exchanger
NES	Nestin
NeuN	Neuronal nuclei
NFAT	Nuclear factor of activated T-cells
NSCs	Neural stem cells
OCT4	Octamer-binding transcription factor 4
OLIG2	Oligodendrodyte transcription factor 2
PMCA	Plasma membrane calcium adenosinetriphosphatase
Rac1	Ras-related C3 botulinum toxin substance 1
RNA	Ribonucleic acid
RYR	Ryanodine receptor
SERCA	Sarco/endoplasmic reticulum calcium adenosinetriphosphatase
shRNA	Short hairpin ribonucleic acid
SOX2	Sex determining region Y-box 2
STAT3	Signal transducer and activator of transcription 3
TMRE	Tetramethylrhodamine ethyl ester
TMZ	Temozolomide
TRP	Transient receptor potential
VGCC	Voltage gated calcium channel
VIM	Vimentin

1 INTRODUCTION

1.1 CANCER STEM CELLS

It has long been established that cancer is driven by the acquisition of various types of genetic alterations such as activation of oncogenes, gene amplification and the repression of tumor suppressors in normal differentiated cells. For many decades, cancer studies have been based on a stochastic model of oncogenesis where all cells have the same probability of becoming a cancer cell via the accumulation of sufficient mutations [1,2]. However, it was always an enigma how terminally differentiated, non-dividing cells are able to accumulate enough mutations to enable continuous replication that can give rise to the bulk tumor. Moreover, most tumors are heterogeneous and contain cells exhibiting different degrees of differentiation. This raises another question of how a differentiated cell is able to divide and generate cells of varying degrees of differentiation. To address these questions, an alternative hypothesis was introduced, which postulates that cancer is driven by a small population of cells known as cancer stem cells (CSCs).

The cancer stem cell hypothesis proposes that a small population of cancer cells possesses stem cell properties of self-renewal and differentiation, and are the underlying drivers of tumor initiation. Together with their more differentiated progeny, CSCs build up and maintain the tumor mass. This cell population is currently defined by functional parameters based on experimental observations. Firstly, these cells must have the ability for long-term self-renewal. This allows the cells to have limitless regenerative potential to support the growth of the tumor. Secondly, the cells must possess the capacity to differentiate, giving rise to tumorigenesis and the heterogeneity that is seen in the tumor. Thirdly, these cells also have the ability to form tumors after xenotransplantation [3–5], and can be expanded in adherent culture whilst maintaining a very high percentage of CSC population [6].

The study of CSCs is a very dynamic field, with constant changes and additions to our understanding as more research is done. The hypothesis that cancer could arise from the activation of dormant stem cells was first proposed by German pathologist Julius Cohnheim in 1875 [7]. This idea was strengthened with the first assay demonstrating the existence of this group of cells *in vitro* [8], and the observation that poorer patient prognosis was seen in germ cell tumors with the presence of undifferentiated cells in the residual tumor masses post-chemotherapy [9]. The first identification and characterization of cancer stem cells was in acute myeloid leukemia in 1997. By transplanting $CD34^{+}CD38^{-}$ fractions from patients into mice, CSCs were able to recapitulate the same leukemic phenotypes seen in the patient [4]. Since then, more evidence of CSCs in different cancers has emerged, such as in multiple myeloma [10], breast cancer [3] and brain cancer [11,12].

Despite the numerous studies, uncertainty still remains about the origins of CSCs and if this model is applicable to all cancer types. One study has shown that two populations of cells exist in squamous skin tumors, and evidence presented that the CSCs drove the development of the bulk tumor [13]. Lineage tracing has also shown that it is the CSC population that

contributes to the formation of the tumor [14]. With regards to the origin of CSCs, it was shown in some cancers that the CSC pool may have originated from normal tissue-resident stem cells that acquired oncogenic mutations that drove self-renewal [15,16]. This led to the formation of different subclones from the parental CSC, all of which continued to contribute to the growth of the bulk tumor [17,18] (Figure 1). Others have identified that CSCs arise from dedifferentiation of differentiated cancer cells due to the influence from the tumor microenvironment [19].

Transplantation experiments in mice have yielded further evidence supporting the existence of CSCs. Transplantation of tumor cells that were pre-sorted to enrich for CSCs showed that they were up to 100-fold more efficient in tumor formation than unsorted bulk tumor cells [3,4,20]. However, the frequency of CSCs is highly variable depending on the type of cancer, and can range from below 1% as seen in acute myeloid leukemia [4], to 25% in melanoma [20]. CSCs are also relatively abundant in some cancers, such as acute lymphoblastic leukemia (ALL), where at least 50% are capable of forming tumor formation in wild type mice [21].

The relative rarity of CSCs has led to the development of protocols for their isolation from solid tumors using various cell surface markers that were enriched in the cancer types. $CD34^{+}CD38^{-}$ fractions determined the presence of CSCs in leukemia [4], while a $CD44^{+}CD24^{-/low}$ profile was used to isolate CSCs from breast cancer [3]. CD133 (Prominin-1) was used as a marker to isolate CSCs from glioblastoma multiforme, colorectal and pancreatic cancers. Despite multiple studies done to show the usefulness of using markers to isolate the CSCs, one has to pay attention to the use of these cell surface markers as a selection tool for CSCs as they may not be representative of the entire CSC pool. A good example of this was seen in leukemia where $CD34^{+}CD38^{-}$ cells were thought to be the CSCs, only for it to be later shown that leukemia CSCs could also be seen in the $CD34^{-}$ and $CD38^{+}$ populations [22–24]. Similarly, breast CSCs that were first identified by their $CD44^{+}CD24^{-/low}$ profile were later identified by different markers such as CD61 [25] and $lin^{-}CD29^{high}CD24^{high}$ [26]. It would appear that a combination of markers has to be employed while isolating these cells from bulk tumors and these markers have to be tested in a large number of patients. In the end, the most conclusive verification method is transplantation to check for tumorigenic property.

Recent advances in genomics have generated large amounts of CSC sequencing data showing that CSCs carry a stem cell signature, which is often a prediction of poor prognosis and high propensity for relapse [27]. When comparing CSCs to stem cells, normal cells and differentiated cancer cells, it was found that CSCs often have a gene expression pattern that is most similar to that of the stem cells, which has been termed the “stem cell signature” [16,24]. This genomic data further demonstrates that CSCs have properties that resemble stem cells, which allow the cells to contribute to tumor initiation and growth, therefore giving CSCs their deadly reputation.

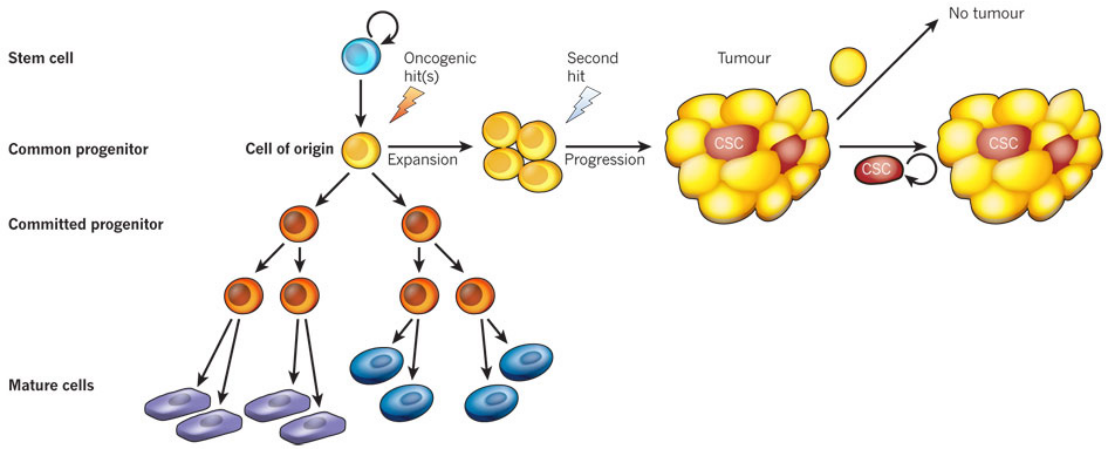

Figure 1. The evolution of cancer stem cells. Normal cellular hierarchy with stem cells that generate more restricted progenitor cells, which eventually give rise to the matured, differentiated cells. The cell of origin for a tumor could arise from an early progenitor cell, where the accumulation of mutations leads to the formation of a CSC. The CSC then continues to drive tumor growth by continuous self-renewal and the generation of differentiated cancer cells. Reprinted with permission from Nature Publishing Group: Nature, Visvader. (2011) [28]

1.2 GLIOMA INITIATING CELLS

Glioma is a common type of brain tumor and has been classified into four different grades based on morphological and histological features. The most malignant form is a grade IV glioma, also known as glioblastoma multiforme (GBM), which is associated with poor prognosis and has an average median survival of 15 months [29]. This high morbidity is in part due to the location of the cancer in the brain, which prevents adequate resection of the tumor. Despite the combination of surgery, chemotherapy and radiotherapy, more than 90% of the patients showed recurrence [30]. The recurrence is believed to be caused by the expansion of a distinct subpopulation of cells within the solid tumor that follows the CSC hypothesis.

This subpopulation of immature cells in the highly lethal cancer, termed glioma initiating cells (GICs) [5,31–33], coexist with the differentiated cell populations in the bulk tumor. In 2003, Singh *et al* described the presence of brain tumor stem cells (BTSCs) in human patient material, including the GICs. This group of cells was isolated based on enrichment for the neural stem cell surface marker CD133. In culture, the $CD133^+$ cells isolated from the tumor were able to form neurosphere-like colonies and could be differentiated into tumor cells that resembled the original patient tumor [5] (Figure 2). The frequency of GICs in GBMs was estimated to range between 19-29%, and as few as 100 $CD133^+$ cells were sufficient to produce tumors in mice, with xenografts exhibiting histopathological phenotypes matching the patient's original tumor [11].

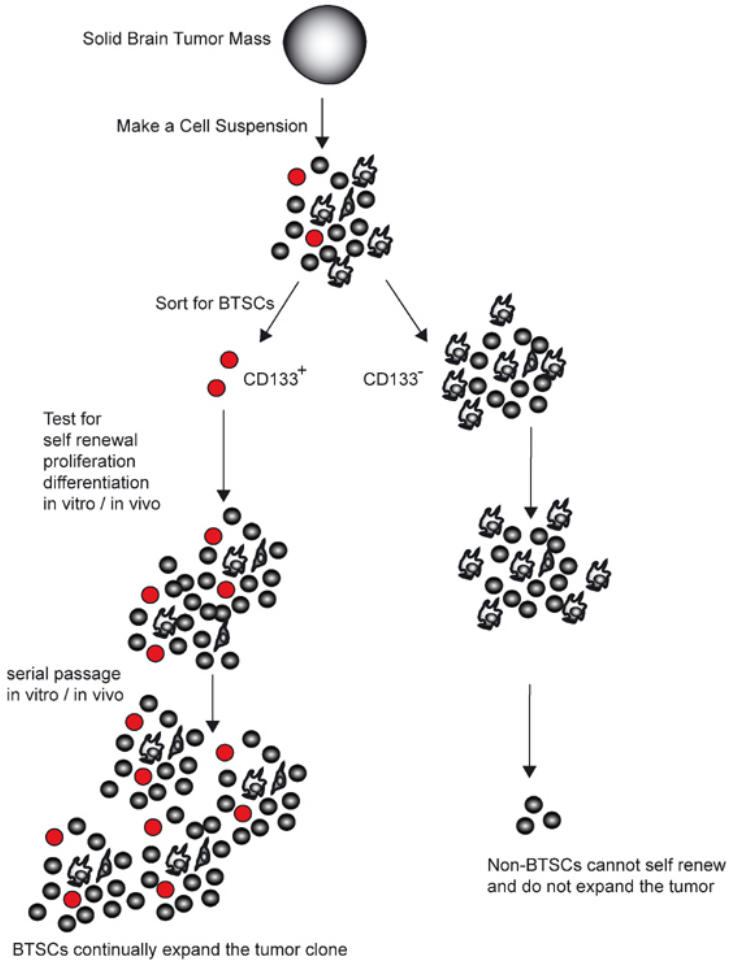

Figure 2. Identification of GICs from solid tumors after dissociation. CD133 is used as a marker to identify the small population of cells within the tumor mass that can uniquely propagate the tumor. Reprinted with permission from Nature Publishing Group: Oncogene, Singh *et al*. (2004) [34]

However, one must be aware of the heterogeneity that exists amongst patients. CD133 received plenty of attention as the marker uniquely identifying GICs [5], and many studies were conducted without any further experimental validation. This was soon proven to be an erroneous generalization when various groups showed that CD133- cells were also capable of forming glioblastoma [35–38]. Moreover, CD133 alone or in combination with other markers, could be used to identify CSCs from other cancers such as prostate [39], lung [40] and liver [41]. Since then, differing opinions have surfaced on the utility of CD133 expression as a good prognostic marker. Some studies have shown that high CD133 expression in patient tumors often leads to poorer prognosis, and is correlated to shorter survival of the patients [42,43]. However, another study has shown that CD133 is actually correlated to longer survival after tumor recurrence [44]. This demonstrates that while CD133 can be used as an indicator of the presence of GICs, it should not be taken to be the one marker for GICs. Further studies have to be done to find out the biological significance of CD133, as well as to assess its proper role in identifying GICs.

GICs can also be established as cell lines, both as spheres [5,31] and adherent cultures [6]. The adherent culture was shown to be a more efficient culture system where GICs could be propagated more stably than in sphere culture. This was attributed to the possibility of spontaneous differentiation and cell death in the sphere culture due to the non-uniform exposure to growth factors, oxygen and nutrients. The adherent culture condition on the other hand could fully suppress the differentiation of the GICs to oligodendrocytes and neurons, as shown by O4 and Tuj-1 stainings respectively. The adherent GICs showed high infiltration into the host brain when transplanted into mice. They also kept their tumorigenic properties and were able to generate tumors that exhibited pseudopalisading necrosis, nuclear polymorphism and microvascular proliferation, which are key histopathological features of human GBM tumors [45].

The question of where do GICs arise from, and how similar they are to stem cells remains unanswered. GICs have been shown to share expression of stemness-associated genes with stem cell populations [27,46]. Functionally, it was demonstrated that GBMs contain tumorigenic neural stem-like cells that could be differentiated to GFAP-positive astrocyte-like cells or Tuj1-positive neuron-like cells [33]. When compared to normal brain tissue, GICs exhibited enrichment in expression of genes normally associated with embryonic stem cells, which includes genes such as Oct4, Musashi and Sox2 [27]. GICs were also shown to have a gene expression profile that was more similar to that of fetal neural stem cells than of adult brain tissue [6], and it is proposed that this group of cells continuously resupply the bulk tumor cells through self-renewal and cell differentiation [6,7]. These observations indicate that GICs may originate from neural stem or progenitor cells, and they carry a stem cell signature that one might use as a predictive tool for prognosis.

Gene expression profiling of gliomas as a predictive tool for survival gave us a more in depth look into the molecular classification of the cancer [47–49]. Gene expression studies showed that genetic signatures exist amongst the different grades of glioma, and gene expression-based clustering of gliomas results in the emergence of groups with different survival and recurrence prognosis. Phillips *et al* first published a panel of molecular subclasses of the glioma samples, and showed that the different subclasses resembled different stages in neurogenesis. Neuronal differentiation markers such as OLIG2, MAP2, NeuN and DCX were expressed in the proneural subclass, while markers of NSCs such as VIM, NES and CD133 were expressed in the mesenchymal and proliferative subclasses. Interestingly, the authors highlighted that it is the mesenchymal and proliferative subtypes that correlated to poorer prognosis. This indicated that signaling pathways involved in the maintenance of neurogenic potential play critical roles in tumor aggressiveness [49]. Verhaak *et al* extrapolated this study by utilizing genomic data from the Cancer Genome Atlas (TCGA) Research Network to identify two additional subtypes: classical and neural. This study yielded four subtypes of GBM that were determined by expression patterns, and this data was compiled and compared to gene expression from a collection of xenografts so as to determine the tumor subtype. The dataset was then compared to gene expression sets from neurons, oligodendrocytes, astrocytes and cultured astroglial cells, where each subtype was shown to be linked to a

possible cell of origin. This led to the current nomenclature for classification: proneural, neural, classical and mesenchymal. Whilst this data supported the finding of the proneural and mesenchymal subclasses as defined by Phillip *et al*, Verhaak *et al* also highlighted alterations in oligodendrocytic development gene PDGFRA as well as higher expression of SOX genes in the proneural signature. In the classical subtype, genes in the Notch and Sonic hedgehog pathway, as well as NES were highly expressed, and there were abnormalities in EGFR expression [50]. The authors also observed that the proneural signature predicted improved survival, which was supported in another independent study [51]. The evidence presented so far suggests that there might be stratification in the cell of origin of GICs, and patient prognosis is correlated with tumor classification into one of the characteristic groups with defining gene expression patterns. This suggests that tumor classification can also be used to help predict responses to therapy.

GICs are highly resistant to chemo- and radiotherapy [52–54]. Radiation of human glioma xenografts, both *in vitro* and *in vivo*, led to the enrichment of the $CD133^+$ population. This population of cells was able to initiate tumor growth upon transplantion into mice, therefore supporting the notion that it is the GICs that are crucial for recurrence [52]. GICs also exhibited resistance to temozolomide (TMZ), a chemotherapeutic agent commonly used for glioma treatment. It was also shown that TMZ affected NSC viability, but had minimal effects on GICs [54]. These properties of GICs prove to be a major challenge for therapies. Moreover, conventional therapies remove the bulk tumor and most of the differentiated glioma cells, whereas the relatively quiescent GICs are able to escape. These remaining GICs are suggested to then initiate the regrowth of the tumor [55], thus leading to relapse in patients.

1.3 TREATMENTS TARGETING GICS

Conventional cancer treatment involves the use of cytotoxic chemotherapeutic agents that target highly proliferative cancer cells. Most of these drugs have a cytostatic effect which block cells in various stages in the cell cycle. Treatment for glioma patients is no exception, and for many years, a combination of surgery, radiotherapy and chemotherapy has been the gold standard of treatment for these patients. Despite such harsh strategies to combat the disease, prognosis remains poor and the median survival remains as low as 14-16 months [29].

Temozolomide (TMZ) is one of the common chemotherapeutic agents used as part of the current regiment to treat GBM patients. It is an alkylating agent that crosslinks DNA, therefore preventing cells from further division. TMZ was rapidly absorbed and showed relatively low toxicity to patients during preclinical studies, which has made TMZ the drug of choice for treatment [56,57]. Despite its effectiveness against differentiated glioma cells [58], GICs have exhibited resistance to TMZ due to their low proliferation rate and quiescent

nature. They are spared by TMZ treatment and can initiate the regrowth of the tumor [55,59,60], which leads to relapse in patients.

Another cause for concern with TMZ treatment is its adverse effects on NSCs. While having no significant effect on GICs, it led to a reduction of NSC viability [54]. Since NSCs exists during childhood and persist during adulthood [61], TMZ treatment causes a chronic loss in neurogenic potential that could lead to detrimental side effects on learning and memory.

Additionally, a recent paper has shown that differentiated glioma cells could go through a phenotypic shift to acquire a GIC-like status upon TMZ treatment [62]. This was characterized by reduced expression of the differentiation marker GFAP and an increase in stem cell markers CD133 and Sox2. This conversion rate was increased upon long term TMZ treatment, and could be yet another plausible explanation for the high rate of recurrence in glioma patients post treatment.

With more studies that show the ineffectiveness of current chemotherapy treatments, there is an urgency to find more effective ways of treating glioma and specifically GICs. Various methods that have been proposed include inducing differentiation, targeting signaling pathways that are involved in GICs maintenance and function, or inhibiting their survival mechanisms. Bone morphogenetic proteins (BMPs) have been shown to induce differentiation in GICs, thereby inhibiting tumorigenicity *in vivo* [63]. The inhibition of Notch signaling was also shown to be effective in inducing differentiation of GICs [64] and reducing radioresistance [65]. Inhibition of STAT3 has been shown to disrupt GIC proliferation, as well as deplete stemness markers [66]. Micro-RNAs have also been suggested to be effective by affecting the apoptotic signaling pathways, which enhanced sensitivity towards chemotherapeutic agents [67].

The discovery of GICs has altered our understanding of glioma tumor biology and has led researchers to reevaluate current therapies. Novel strategies targeting GICs are being developed, and more care is taken to understand the similarities and differences between GICs and NSCs so as to develop drugs that can specifically target the former cell type.

1.4 CHEMICAL SCREENS – PARAMETERS USED

High throughput chemical screening is a relatively fast and effective way to discover possible hit or lead compounds that shows efficacy against different diseases, including GBM and GICs. There are various parameters that one could consider when doing a screen. In the most ideal setup for drug screens, a uniform layer of cells are exposed to equivalent quantities of drugs in medium that does not hinder binding efficacy nor affect the rate of metabolism of the drug. Some clinically relevant parameters are then used as a readout to assess drug potency. Cancer cell survival is often used as a readout by measuring cell viability, which is the quantification of ATP amounts in a cell. It has proven to be fast and accurate in deriving dose responses.

The above requirements make neurosphere GIC cultures poorly amenable to chemical screening. The 3D growth results in uneven drug exposure, and spontaneous differentiation and cell death in each sphere adds noise and may result in false-positive data. However, Pollard *et al* made drug screening for phenotypes in GICs tractable with their adherent culture protocols in 2009 [6]. They managed to maintain a relatively pure and stable culture that exhibited no major changes from the parental population in early passages, and have suggested that each GIC line be expanded for no more than 20 passages.

Following identification of a potential hit compound, the mechanism of cell death is often looked. This gives us an understanding of the signaling pathways that could be involved, as well as determine if the drug might be useful in clinical trials. Listed below are brief overviews of the different types of cell death classified into two groups based on today's nomenclature: conventional (programmed) and non-conventional.

1.4.1 Conventional (programmed) cell death

Apoptosis is a type of programmed cell death that leads to characteristic morphological changes such as cell rounding and shrinkage, nuclear fragmentation, chromatin condensation and membrane blebbing [68]. Different signaling cascades are implicated: for example the involvement of death receptors such as FAS, the involvement of BCL-2 family members in mitochondrial outer membrane permeabilization or the involvement of cytochrome c due to sustained elevated levels of calcium (Ca^{2+}), all of these finally lead to the activation of downstream caspase cascade that is then the effector of apoptosis [69]. Caspase-independent apoptosis has also been shown to occur by inhibiting ATP synthesis or the respiratory chain, or by increasing ROS production [70]. Apoptosis markers such as cleaved caspase-3 and annexin-V are commonly used in screens.

Outside of the mitochondria, which are widely known to be key in apoptosis induction, the endoplasmic reticulum (ER) has also been established to partake in apoptosis. It is widely known to be an internal Ca^{2+} store, and it was shown that BCL-2 is able to regulate Ca^{2+} efflux from the ER, thereby controlling the onset of apoptosis [71]. Moreover, the ER plays an important role in Ca^{2+} homeostasis by taking up excess intracellular Ca^{2+}. Any aberration of this process will lead to a deregulation of Ca^{2+} homeostasis and drive the cells to apoptosis [72] (Figure 3).

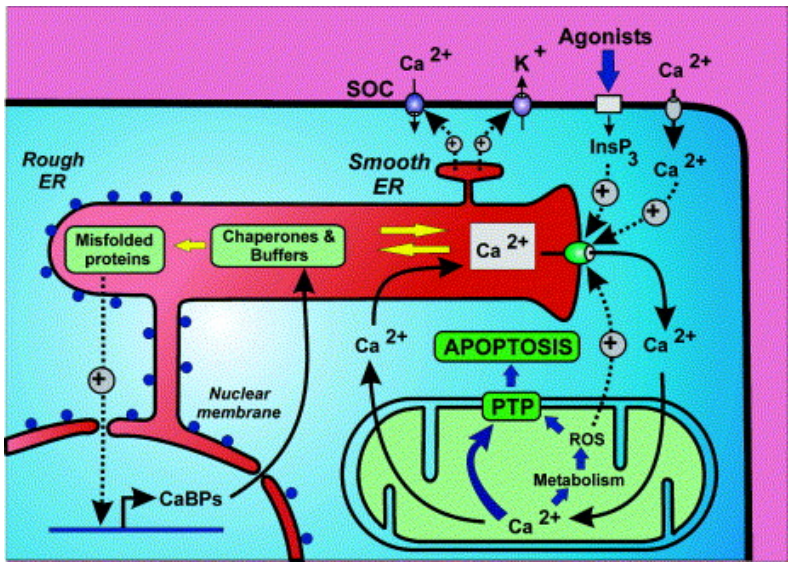

Figure 3. Involvement of ER in apoptosis. Reprinted with permission from Elsevier: Cell Calcium, Berridge (2002) [73]

Autophagy was initially thought to have a pro-survival role in cell homeostasis that sets in during periods of starvation and stress. It is a catabolic process that allows the cell to "ingest" its own organelles via digestion within double membrane-bound structures known as autophagosomes [74]. However, accumulating evidence shows that if cells are unable to cope with the stress, autophagic cell death can also occur [75,76]. It is often accompanied by the presence of modulators such as members of the ATG family [77]. ATG8, or the mammalian homolog LC3, is commonly used as a readout for autophagic activity in most assays, including high-throughput screens [78,79].

Necrosis has long been thought to be an accidental type of cell death that is not controlled by any molecular mechanisms. It is morphologically characterized by cell swelling, organelle dysfunction and cell lysis [80]. However, recent evidence has shown that necrosis is intricately regulated by various signaling pathways, including the activation of serine/threonine kinases [81].

From a therapeutic perspective, necrosis is not a preferred choice of cell death as the release of the cell contents will cause immune cells to release pro-inflammatory cytokines [82], which triggers inflammation and may further exacerbate the condition. In contrast, cell membrane integrity is maintained during apoptosis, and apoptotic cells are cleared by phagocytes [83], therefore immunogenic response will not being elicited [84]. Similarly, autophagic cell death has been shown to reduce inflammatory reactions [85] and inhibit pro-inflammatory signaling [86].

1.4.2 Non-conventional cell death

Paper I in this thesis revealed an example of non-conventional cell death by macropinocytosis. Macropinocytosis is the process of absorbing extracellular fluids, solutes

and small particles on the plasma membrane via the formation of large uncoated vacuoles [87]. It is a transiently triggered process and operates for a limited period of time [88]. Macropinocytosis has been observed since the 1970s in human glial cells upon the addition of growth factors [89]. It was only in 2008 that a comprehensive study showed macropinocytosis as a form of cell death due to the activation of Ras in glioblastoma cells [90]. In this study, electron-lucent vacuoles were observed upon active Ras expression with the use of electron microscopy. These vacuoles were further distinguished from autophagosomes by the lack of expression of the autophagy marker LC3. There was also no activation of apoptotic markers, yet this process led to eventual cell death by a massive increase in fluid uptake, resulting in cell swelling and membrane rupture. This novel non-apoptotic type of cell death was coined as methuosis.

A follow-up study showed that methuosis could be induced by small molecules. The group did a screen based on compounds that induced cell vacuolization, and the hit compounds exhibited the phenotypic characteristics of methuosis. It was also revealed that Rab5 and Rab7, genes that are involved in the endosomal trafficking pathway, were implicated in the process. A significant decrease of Rab5 was observed, followed by an increase in Rab7 activity upon treatment, suggesting that the compound might be targeting proteins in the endosomal complex, thereby allowing the vesicles formed to bypass the normal endosomal trafficking pathway. The vesicles accumulate and undergo fusion, eventually filling up the cell, impairing metabolic functions and causing cell death [91] (Figure 4).

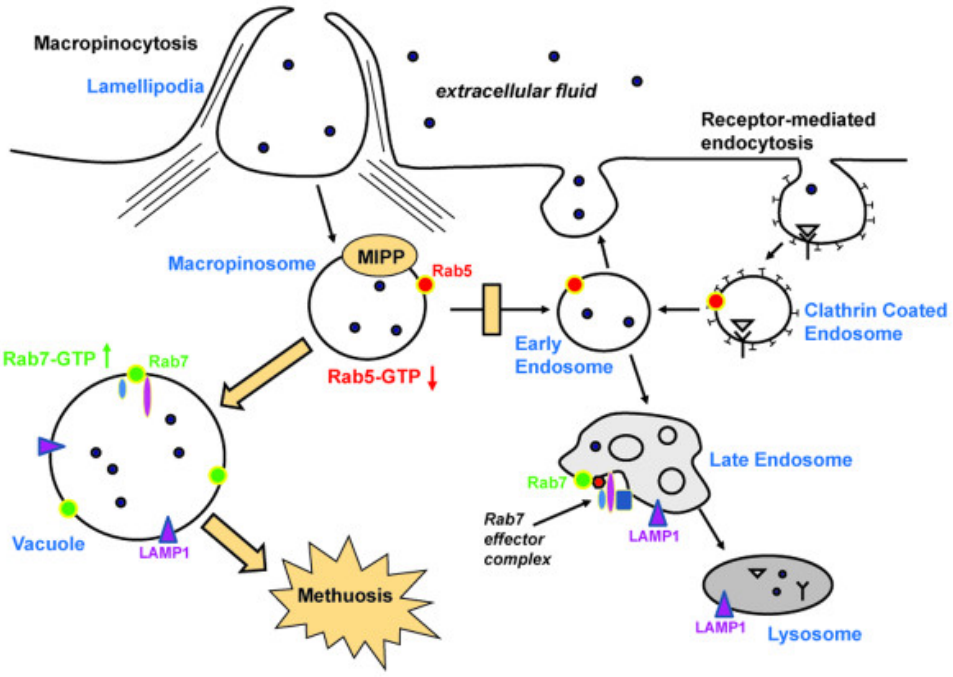

Figure 4. Schematic of the induction of methuosis. This abnormal form of macropinocytosis lacks the components for normal fusion with lysosomes, therefore leading to the accumulation of vacuoles which fill the cell and disrupts membrane integrity. Reprinted with permission from BioMed Central Ltd.: Molecular Cancer, Overmeyer *et al*. (2011) [91]

In Paper I, we have shown that mitogen-activated protein kinase kinase 4 (MKK4) activation was implicated in the formation of macropinocytosis. MKK4 is a member of the MAP kinase kinase family, and directly phosphorylates c-Jun N-terminal kinases (JNK) to elicit various types of cellular responses such as inflammation, apoptosis, growth and differentiation [92]. MKK4 is also involved in the p38 cell stress pathway, resulting in the activation of transcription factors that control functions such as cytokine production and apoptosis [93].

Methuosis is a novel concept of cell death, and the underlying molecular signaling and mechanisms are still not fully understood. It is still left to be identified if methuosis is induced in a controlled manner via specific targets, and if so, what the identities of these targets might be.

1.5 ION HOMEOSTASIS

Ion homeostasis is the process of maintaining the concentrations of cations and anions at a steady-state level in a cell, and this is governed by the actions of ion channels, pumps and transporters. Ion channels are one of the fundamental groups of proteins that are found on every cell. They have long been assigned the role of governing basic cellular processes such as establishing a resting membrane potential, maintaining intracellular osmolarity, regulating cell volume and shaping action potentials in excitable cells. Recently, they have also been shown to control cell cycle in embryonic stem (ES) cells and neural stem cells (NSCs) [94]. For example, ion flux through potassium and calcium (Ca^{2+}) channels has been shown to control diverse functions as proliferation and migration in stem cells and cancer cell lines, including glioma [95,96].

1.5.1 Calcium

Ca^{2+} is known to be a very versatile intracellular signal molecule that can effect and regulate many different cellular processes. Even in non-excitable cells, a 20,000 fold gradient exists between the intracellular and extracellular concentrations of Ca^{2+}, where upon stimulation, the levels of intracellular Ca^{2+} can increase from approximately 100 nM to > 1 μM [97]. Moreover, the life and death choices of the cell hang on a very delicate balance in the levels of intracellular Ca^{2+}. With such complexity and variety in signaling and functions, it is necessary for the cell to exert tight control over Ca^{2+} levels.

One way of achieving Ca^{2+} homeostasis is via the compartmentalization and storage of Ca^{2+} into intracellular organelles, such as the endoplasmic reticulum (ER) and mitochondria. Such methods of storing Ca^{2+} allows for rapid and immediate release of Ca^{2+} when necessary, as well as allowing rapid uptake of Ca^{2+} when the cell is met with a sudden rise in Ca^{2+} levels, therefore preventing any irreversible damage to cellular function. Ca^{2+} stores also have very different functions. Mitochondrial Ca^{2+} has been shown to be a key regulator of both metabolism [98,99] and apoptosis [100], whereas Ca^{2+} from the ER is mainly involved in regulation of apoptosis [72].

Ca^{2+} homeostasis is a highly coordinated process that involves a large number of signaling components. It can be summarized into four different units (Figure 5):

1. Signaling – The conversion of stimuli to Ca^{2+} signals.
2. ON mechanism – Ca^{2+} influx.
3. Decoding – Ca^{2+}-sensitive processes triggered to elicit physiological responses.
4. OFF mechanism – Removal of Ca^{2+} and restoration of basal intracellular Ca^{2+} levels.

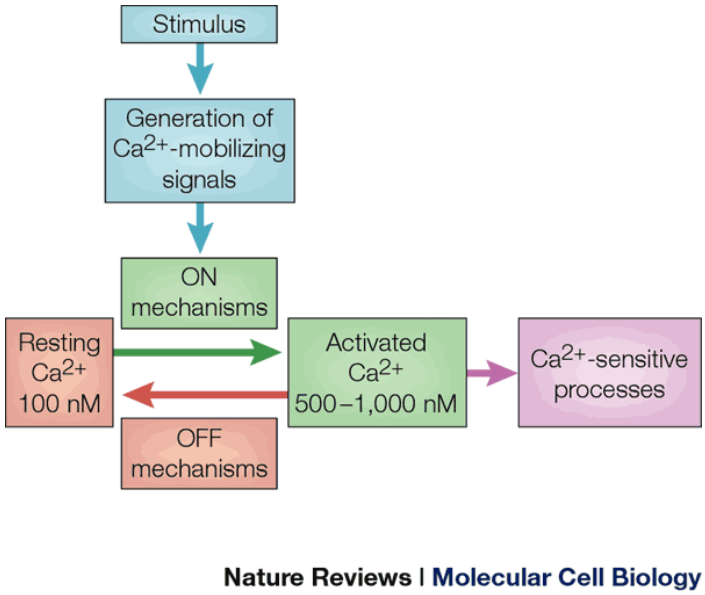

Figure 5. The four units of Ca^{2+} homeostasis. Reprinted with permission from Nature Publishing Group: Nature Reviews Molecular Cell Biology, Berridge *et al*. (2000) [101]

1.5.1.1 Signaling

A diverse range of stimuli binds to various cell surface receptors and trigger the start of Ca^{2+} signaling by different second messengers. One example is the activation of inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$), which in turn binds to inositol-1,4,5-triphosphate receptor ($InsP_3R$) to induce the release of Ca^{2+} from the ER [102]. This will initiate the ON mechanism.

1.5.1.2 ON mechanism

The ON mechanism is induced by an elevation of intracellular Ca^{2+} levels, which is supported from two different groups of sources. Firstly, external sources of Ca^{2+} can be introduced into the cell via different classes of Ca^{2+} channels, such as the voltage-gated Ca^{2+} channels (VGCCs) or receptor-operated channels. For example, ionotropic glutamate receptors are able to induce Ca^{2+} influx in the presence of glutamate [103]. Secondly, internal sources of Ca^{2+} come from the organelle stores such as the ER and mitochondria. Ca^{2+} release from the ER is mainly facilitated by the $InsP_3R$ and ryanodine receptor (RYR) families [104].

1.5.1.3 Decoding

Various Ca^{2+} binding proteins are required to "translate" the Ca^{2+} signals to physiological responses. Two groups of Ca^{2+} binding proteins exist: effectors and buffers. They have opposing effects, and it is the balance between the groups that controls the various cellular processes [105].

The Ca^{2+} binding then evokes gene transcription via Ca^{2+}-dependent transcription factors that are activated by different mechanisms. One example is the activation of nuclear factor of activated T cell (NFAT) by repetitive Ca^{2+} spikings [106], which is known to effect a variety of downstream processes, such as immune response and the development of skeletal muscles [107]. The Ca^{2+} dependent transcriptional changes are also implicated in differentiation in various cell types, including neural cells [108,109].

1.5.1.4 OFF mechanism

Once the desired signal is achieved, basal Ca^{2+} levels are restored by removing Ca^{2+} through different exchangers and pumps. This includes the Na^{+}/Ca^{2+} exchanger (NCX), plasma membrane Ca^{2+}-ATPase (PMCA) and sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). Both NCX and PMCA extrude Ca^{2+} out of the cell, while SERCA pumps Ca^{2+} back into the ER [97].

One way of studying the effects of Ca^{2+} homeostasis is to employ compounds that target the different Ca^{2+} ion channels and pumps. Below is a general overview of the three different types of compounds that we have used in Papers II and III, and their different modes of action.

1.5.2 A23187

A23187 is a well-known carboxylic acid antibiotic that plays the role of a Ca^{2+} ionophore [110]. The compound forms a dimer that complexes with Ca^{2+} and transports the ion through the plasma membrane [111], resulting in a rapid increase in intracellular levels of Ca^{2+} [112]. This leads to the depletion of Ca^{2+} from the intracellular stores [113], which activates plasma membrane Ca^{2+} channels to allow an influx of Ca^{2+} into the cell [114]. The sustained high levels of Ca^{2+} in the cells were thus maintained for periods of time, resulting in apoptosis.

1.5.3 Thapsigargin

Thapsigargin is another well-established compound that affects Ca^{2+} homeostasis, in this case by irreversibly inhibiting the SERCA pumps [115]. This raises the levels of intracellular Ca^{2+} by blocking the cell's ability to pump Ca^{2+} back into the ER, and more importantly, causes the depletion of Ca^{2+} stores, which similarly to A23187, activates plasma membrane Ca^{2+} channels to allow influx of Ca^{2+} into the cell [114]. This disruption to Ca^{2+} homeostasis drives the cell into apoptosis.

1.5.4 Niguldipine

Niguldipine is classified under the group dihydropyridines, and was discovered to be an antihypertensive agent due to its vasodilator activity [116,117]. This activity was due to its binding to the T- and L-type VGCCs [118] and alpha 1-adrenoceptors [119]. More recently, Niguldipine has been shown to induce autophagy in glioma cells [79], and can cause a reduction of cell viability to GICs in our study, as shown in Paper III. This shows that there is much potential in old pharmaceutical compounds, which is reflected in the current trend to reposition these compounds in cancer therapy.

1.5.5 Involvement in cancer

In cancer research, Ca^{2+} has been implicated in a variety of processes, including cancer cell survival [120]. Due to the intricate nature of the balance of intracellular Ca^{2+}, researchers have been exploring with the idea of driving cancer cells to choose the death pathway by influencing intracellular Ca^{2+} levels. It was recently shown that interference with a Ca^{2+} channel subunit was able to drive liver tumor-initiating cells into apoptosis [121].

Due to the wide spectrum of signaling pathways Ca^{2+} is involved in, the deregulation of Ca^{2+} homeostasis can also cause tumor progression. Cancers have been shown to carry altered expression of Ca^{2+} related genes, with some of the alterations being correlated with prognosis. Many types of Ca^{2+} channels have aberrant expression in cancer cells. One example is the transient receptor potential (TRP) channels, a superfamily of Ca^{2+} channels. Inhibition of TRP channel activity suppressed glioma growth [122], and increased expression has been observed in prostate [123], colon [124] and bladder cancer [125]. Another example is seen in the overexpression of T-type VGCC that resulted in increased cell proliferation in glioma [126] and esophageal carcinomas [127]. Aside from Ca^{2+} channels, overexpression and aberrant activation of the Ca^{2+} dependent transcription factor NFAT have also been observed to play a role in tumor progression in various types of cancer, including pancreatic [128], breast [129] and melanoma [130]. NFAT has also been implicated in promoting angiogenesis [131].

With a plethora of effects, targeting Ca^{2+} related genes and proteins in cancer therapy have garnered plenty of interest as more evidence established the importance of Ca^{2+} homeostasis. Strategies involve driving cancer cells towards differentiation, inhibiting cell proliferation and increasing sensitivity to apoptosis by controlling and affecting the Ca^{2+} pathways. However, due to the ubiquitous behavior of Ca^{2+} signaling, selective targeting of components in cancer cell and CSCs remain to be a major challenge.

Proliferation of glioma cells has been shown to be inhibited by the L- and T-type channel blocker mibefradil, and the cells are driven to apoptosis [132,133]. Mibefradil also increased survival when used together with conventional treatments such as radiosurgery *in vivo* [134], as well as sensitized TMZ resistant glioma cells, enhancing the efficacy of TMZ [135]. It remains to be seen if this combination of treatments is able to overcome the resistance seen in GICs to TMZ treatment [53].

The idea of using ion channel modulation as a form of therapy for targeting GICs has only recently emerged. The relationship between ion channels and GICs remains obscure, however present studies imply that the expression of ion channels and Ca^{2+} related genes are abnormal in GICs. This idea was corroborated by our findings in Paper II and III.

2 AIMS

The overall aim of this thesis was to find small molecules that can be used as potential treatments against GICs, and to try to understand how these agents work in GICs.

The specific aims were:

• To find compounds that target GICs via a small-to-medium drug screen.

• To investigate how ion channel antagonists and inhibitors affect GIC viability as compared to their differentiated counterparts and normal cells.

• To investigate how ion fluxes, specifically those essential to Ca^{2+} homeostasis, could effect GIC viability.

3 RESULTS AND DISCUSSION

3.1 PAPER I

3.1.1 Small molecule screening revealed rapid and specific GIC death induced by Vacquinol-1

Small molecule screens have often been used to find potential hit compounds targeting cancer cells, however the screens are usually more simple, looking at only one or a couple of aspects, such as: 1) cell death monitored with the use of caspase 3 or Annexin V; 2) autophagy using LC3; or 3) cell viability using an ATP or MTT based assay. In this paper, we have conducted a screen covering all the above mentioned parameters, and sieved out the hit compounds that were seen to affect the GICs only, yet spare all normal cells such as mouse ES cells (mESC) and human fibroblasts.

We started with a library of 1364 compounds and conducted a phenotypic screen on the GICs, which rendered 234 compounds. Further counter screens using mECSs and human fibroblasts were done to identify 63 compounds that had an effect on GICs only. We then used a recovery assay to filter out compounds that caused an irreversible effect on the GICs, combined with in silico absorption, distribution, metabolism and excretion (ADME) prediction, we were left with 17 potential compounds, which were then thoroughly examined using the following assays: 1) Selectivity was tested by setting up a hanging drop-based mixed culture using GICs and fibroblasts; 2) Toxicity was tested by administering the drugs to zebrafish embryos; 3) Cardiovascular toxicity was checked using ex vivo zebrafish hearts and 4) *in vivo* efficacy was monitored by xenotransplanting labeled GICs into zebrafish. This battery of thorough testing revealed Vacquinol-1, which was a potent drug that passed all criteria necessary if one would like to push this compound into clinical trials. In summary, Vacquinol-1 displayed high cytotoxicity, significantly reduced GIC viability and was able to selectively target GICs.

3.1.2 Vacquinol-1 induced cell death via an unconventional pathway

We proceeded to check for the mechanism of cell death, and discovered that Vacquinol-1 did not act via the apoptotic pathway. Apoptosis inhibitor Q-VAD only modestly rescued cell death in Vacquinol-1 treated cells, and Vacquinol-1 did not elevate levels of cleaved caspase 3, nor caspase 3 and caspase 7 enzymatic activity. We also checked if GIC death was caused by mitochondrial death pathway. Tetramethylrhodamine ethyl ester (TMRE) incorporation was not increased, indicating that there was no disruption in the mitochondria. Autophagy-associated cell death was also ruled out as there was no increase in LC3-II in GICs upon treatment.

Vacquinol-1 treatment led to an increase in vacuole formation, whereby vacuole numbers increased in a dose-dependent manner. The vacuoles were a result of macropinocytosis, as shown by increased uptake of the tracer lucifer yellow. This uptake was significantly reduced when the cells were co-treated with drugs such as Bafilomycin A1, Dynasore and

Cytochalasin D, which have been shown to perturb macropinocytosis activity. Further transmission electron microscopy analysis confirmed massive vacuolization in cells, as well as swollen ER and mitochondria and distorted nuclear membrane structures. The vacuoles formed by Vacquinol-1 treatment were also shown to recruit the late endosomal and lysosomal marker, lysosomal-associated membrane protein (LAMP1). These provided evidence that Vacquinol-1 induced massive macropinocytosis, leading to catastrophic vacuolization and a necrotic-like cell death.

We used a shRNA screen to identify the pathways involved in vacuolization, and found that MKK4 had a critical role to play in the process. Vacquinol-1 treatment led to a rapid activation of MKK4, and knocking down the gene (MAP2K4) in GICs led to a huge reduction in uptake of lucifer yellow upon Vacquinol-1 treatment. Although the involvement of MKK4 activation in macropinocytosis is not fully understood, one can speculate of its involvement downstream of Ras-activated Rac1, which is induced during periods of cellular stress. These are pathways that have been implicated in apoptosis [136] and autophagy [137], and due to the complexity and cross-talk between the different pathways [138], it could be speculated that the response machineries of methuosis share common signaling pathways with the more well established death pathways.

3.1.3 Vacquinol-1 led to the attenuation of tumor growth *in vivo* and prolonged survival

Zebrafish xenograft glioblastoma model showed that the GICs could rapidly expand to form a tumor mass that started to infiltrate into the brain, which was stopped with Vacquinol-1 treatment. In the mouse xenograft glioblastoma model, similarly, all the mice presented large and highly vascularized tumors that infiltrated into the brain, with the presence of massive areas of necrosis in 7 weeks. Vacquinol-1 was administered intracranially only 6 weeks after engraftment, and despite the advanced stage of GBM, the whole brain showed improved morphology with a smaller area of necrosis. There was increased LAMP1 staining in the tumor cells of Vacquinol-1 treated mice, showing that a similar initiation of macropinocytosis and the activation of methuosis occured *in vivo*. The effective dose of Vacquinol-1 also had no effect on the host brain, showing that the drug has the capacity to attenuate tumor growth *in vivo*.

We then explored the bioavailability of Vacquinol-1 *in vivo* via oral, intravenous and intraperitoneal administration, and showed that Vacquinol-1 showed good penetrance into the brain, had maximal plasma exposure and was highly stable *in vivo* with very low systemic plasma clearance. This suggested that Vacquinol-1 could be administered orally, and we showed that the oral route of administration did not diminish the therapeutic effects of Vacquinol-1. Even when treated orally, the mice showed normal brain morphology, had a normalized brain weight and vastly improved survival, where only two out of the eight

Vacquinol-1 treated mice died during the 80 days of experiment. This is a vast improvement as compared to vehicle-treated mice, where median survival was only 31.5 days.

The screen has led to the identification of a small molecule that can selectively target GICs by initiating non-apoptosis related cell death. This novel way of cell death by the induction of macropinocytosis, otherwise known as methuosis, did not lead to any adverse side effects in the mice during treatment. This could be another preferred method of inducing cell death in cancer treatments. With all the evidence presented, Vacquinol-1 shows high potential for clinical trials.

3.2 PAPER II

3.2.1 Differential expression of Ca^{2+} homeostatic genes identified in GICs

With the ease and availability of RNA sequencing, global gene expression, or transcriptome studies, have been incorporated into glioma research. It is known that GBM is a heterogeneous disease, where each patient's tumor and condition is different. To be able to classify GBM into different subtypes, different research groups have embarked on the study of gene expression [6,49,50,139]. The cells that were used by Pollard *et al* have also been investigated using microarray. However, microarray data displays high background and is not deep enough to uncover different isoforms and genes, thereby resulting in a loss of vital information.

From our RNA sequencing data, we have uncovered a plethora of genes that were not picked up by the microarray done by Pollard *et al*. These genes are mainly involved in Ca^{2+} ion binding and channel activity. As mentioned by Berridge *et al*. [140], Ca^{2+} homeostasis is maintained by two groups of proteins – effectors and buffers. Our data revealed that GICs could be split into two groups – NSC-proximal GICs and NSC-distal GICs. The NSC-proximal GICs had a higher expression of the effectors, whereas the NSC-distal GICs had a higher expression of Ca^{2+} buffers, which we have also corroborated by western blot analysis of the Ca^{2+} effector GRIA1 and the Ca^{2+} buffer S100A6.

3.2.2 GIC sensitivity to Ca^{2+} modulators followed transcriptome rank order relative to NSCs

To confirm the gene expression analysis, we tested two Ca^{2+} modulators on different GIC cell lines. Both the antibiotic A23187 and the SERCA pump inhibitor Thapsigargin treatments revealed a sensitivity rank order that was based on the GIC cell line's proximity to NSC in gene expression, where the NSC-distal GIC lines were less sensitive to the Ca^{2+} perturbations caused by the drugs.

We selected A23187 it should not be affected by gene expression, since it is an ionophore which can physically manipulate the cell membrane to allow entry of Ca^{2+} ions.

We further investigated if this trend would extend to the more differentiated end of the spectrum by using serum-differentiated GICs. Upon treating the differentiated GICs with A23187, we discovered that the effects on cell viability were not as severe as compared to their undifferentiated counterparts, which further corroborated our earlier finding.

3.2.3 GIC sensitivity rank order to Ca^{2+} modulators correlated to NES, BLBP and GRIA1 expressions

Correlation analysis of NSC marker gene expression and sensitivity to Thapsigargin treatment was performed in nine additional GIC lines, revealing that both NES and BLBP were linked to the elevated sensitivity to Ca^{2+} seen in GICs. The analysis also corroborated our earlier finding that GRIA1 is also correlated to GIC sensitivity to Ca^{2+}.

Further correlation analysis of genome wide mRNA levels and sensitivity to Thapsigargin of the nine GIC lines led to the identification of a further 785 correlated genes. Gene ontology analysis identified the commonality between these to be their involvement in Ca^{2+}-mediated signaling.

In order to improve our stringency levels, we set a higher threshold on the Thapsigargin sensitivity in the correlation analysis and had 385 genes in this data set. To further stratify the genes that are related to stemness, more stringent filters were applied by first comparing this data set to genes that were upregulated in the NSC-proximal GIC line, followed by another filtering process by comparing the genes to genes that were downregulated after differentiation. Nine genes were identified in this process, where three genes, including GRIA1, were linked to Ca^{2+} homeostasis.

3.2.4 Drug reactome analysis identified Ca^{2+}-induced gene expression

We then decided to identify the underlying differences that resulted in differential intracellular Ca^{2+} responses to perturbations in the GICs. We selected the NSC-proximal GIC line, GliNS1 and the most NSC-distal GIC line, G166NS, and treated them with A23187 for 7 hours. The time was sufficient to elicit the first few waves of transcriptional events [141]. We plotted the genes with altered expression after drug exposure against the mean expression value before drug exposure to identify robustly altered genes with potential biological significance. A very similar set of genes was altered in both cell lines, including Ca^{2+}–binding genes such as HSPA5 and HSP90B1, and Ca^{2+}–related ER stress response genes such as HERPUD1 and CALR. The Ca^{2+}–activated transcription factor NFATC2 was also clearly induced in both lines. However, GliNS1 clearly induced a more significant transcriptome fold change than G166NS, suggesting that there is a more potent onset of Ca^{2+} signaling in the NSC-proximal GIC line.

Interestingly, a set of anti-apoptotic genes was upregulated in G166NS. This suggests that, in addition to the lower sensitivity to Ca^{2+} perturbations, the NSC-distal GIC line could be better equipped to cope with external stress factors and is therefore less sensitive to the drugs used, including the apoptosis inducer Thapsigargin.

Moreover, since Ca^{2+} has been implicated in differentiation and in the state of stemness of stem cells, one could speculate that different levels of Ca^{2+} homeostasis reflect a stemness hierarchy based on the correlation of Ca^{2+} sensitivity to Nestin.

3.3 PAPER III

3.3.1 Chemical library screen revealed GIC sensitivity towards Ca^{2+} modulators

We performed a chemical library screen using 72 ion channel modulators testing their effects on GIC viability. We obtained 11 hit compounds, where 10 of them were Ca^{2+} channel modulators. This further strengthened our finding in Paper II, where GICs were also shown to be sensitive to Ca^{2+} perturbations.

3.3.2 Niguldipine is selective for undifferentiated GICs

Niguldipine was among the ten Ca^{2+} channel modulating compounds. It was observed to have a less severe effect on differentiated GICs, normal NSCs and human fibroblasts as compared to undifferentiated GICs.

There also seems to be a small therapeutic window where Niguldipine has a less toxic effect on NSCs as compared to the GICs. This would be an important factor for drug therapy against GBM, especially in children. Current treatment stratergies of pediatric brain tumors often impede the normal neural development of children, as the drugs used in chemotherapy ends up affecting normal cells, including NSCs. This implies that Niguldipine is a potential lead drug that could be further exploited to create a new analog.

3.3.3 Effects of Niguldipine is not via α-adrenergic receptor signaling

Dihydropyridines like Niguldipine are notorious to have polypharmalogical profiles. It has long been established that Niguldipine is a α-adrenergic receptor blocker, hence we decided to investigate if the effects on cell viability could be due to the α-adrenergic receptor signaling pathway. We treated the GICs with a panel of known adrenergic receptor antagonists that included the selective α1-adrenergic receptor antagonists, Prazosin hydrochloride and Terazosin hydrochloride. All the drugs except Prazosin hydrochloride had no effect on GIC viability, strongly suggesting that the effects elicited by Niguldipine were not via α-adrenergic receptor signaling.

Interestingly, seven of the hit compounds were known to affect VGCCs, including Niguldipine. This strongly suggests that the VGCCs might have a role to play in affecting GIC viability. Since there is widespread expression of the CACNA1 family genes in GICs, it is difficult to pinpoint the specific VGCC that plays a central role in controlling GIC survival. We propose that it could be a concerted effect from multiple VGCCs, combined with the fact that, as we showed in Paper II, GICs are more sensitive to Ca^{2+} perturbations. This

combination leads to an imbalance in Ca^{2+} homeostasis, the inability of GICs to cope with stress, and eventual cell death.

One possible mechanism of cell death is via nutrient starvation that is caused by the deregulation of ion dependent transport due to imbalance in ion fluxes initiated by disturbed Ca^{2+} homeostasis. We have shown that uptake of glutamate, which can be transported into the cell by the sodium dependent excitatory amino-acid transporter (EAATs), was affected upon treatment with some of our hit compounds, including Niguldipine. It has been shown that EAATs can be affected by the NCX [142], which in this case is affected by the Ca^{2+} fluxes.

3.3.4 Niguldipine reduced tumor cell numbers and improved survival rates while having no adverse effect on cardiac rhythmicity

Following the footsteps of Paper I, we proceeded to test if Niguldipine has an effect on cardiac rhythmicity by using a zebrafish ex vivo cardiac assay. We ruled out any side effects on the heart with Niguldipine as it did not affect the heart rhythm. The zebrafish xenograft glioblastoma model showed that Niguldipine could stop the formation of tumor mass of the GICs.

In the murine xenograft model, Niguldipine treatment resulted in a significant improvement in median survival of the drug treated group as compared to the vehicle treated group. The evidence that we have gathered suggests that Niguldipine, which is well known as an anti-hypertension drug, has the potential to be repositioned as a compound that targets GICs.

The sensitivity to Ca^{2+} perturbations in GICs that we have seen in Paper II and III suggest that the difference in expression of Ca^{2+} related genes in malignant and normal cells have a significant contribution to the survival of GICs. Since Ca^{2+} is intricately linked to cell cycle, proliferation and death, and has been shown to oscillate during cell cycle in ES cells [143], it would be no surprise that GICs would have a Ca^{2+} homeostatic profile that is more similar to that of stem cells than differentiated cells.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

From the work presented in this thesis, we can conclude that:

1. Drug repositioning – known small molecules may have an potential as therapy against GICs.
2. Vacquinol-1, A23187, Thapsigargin and Niguldipine are compounds that have the ability to kill GICs.
3. Vacquinol-1 treatment caused the onset of methuosis, a novel kind of non-apoptotic cell death.
4. A stemness associated Ca^{2+} sensitivity exists in GICs, and can be used to target this group of cells by perturbing cytosolic Ca^{2+} levels via channel blockers and inhibitors.

With the identification of GICs, there is now a scramble to search for small molecules that can target this group of cells in hope of improving therapy for GBM patients and reducing the recurrence and mortality rate. We have identified a few compounds that exhibit selectivity towards GICs, with three of them being tied to Ca^{2+} homeostasis. Further work will be needed to determine if more ion channel modulators have the potential to be used for GBM treatment, as well as to explore the mechanisms involved. It will be interesting to determine if the level of stemness is directly coupled to Ca^{2+} sensitivity. Validation *in vivo* is also necessary for the three compounds before one is able to assess their potential for clinical trials. In summary, the search for a viable therapy against GBM and GICs, whether together with the conventional methods or as an adjuvant therapy, is still ongoing.

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* Denotes equal contribution

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