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Evaluation of Novel Drug Candidates Identified by Omics Approaches Against the Glioblastoma Invasive Margin

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Abstract

Glioblastoma is an aggressive and fatal brain tumour, with disease recurrence inevitable despite multimodal treatment. The tumour region associated with recurrence is known as the 'invasive margin', and further research into the molecular and cellular basis of this region is needed to prevent or delay recurrence and improve patient survival. Omics methods enable large-scale investigation of an organism's biological makeup, offering the potential to identify therapeutic targets. This project aims to identify cytotoxic therapeutics against omics-derived glioblastoma invasive margin targets, as well as identify potential protein targets in the invasive margin. *In silico* molecular docking identified idelalisib, domperidone, carvedilol, nilotinib, darifenacin, and lurasidone as promising therapeutic candidates against omics-derived targets. Presto Blue™ viability assays revealed six cytotoxic candidates: domperidone ($IC_{50} \sim 57.41 \mu M$), carvedilol ($IC_{50} \sim 16.96 \mu M$), nilotinib ($IC_{50} \sim 25.37 \mu M$), darifenacin ($IC_{50} \sim 46.77 \mu M$), RSM932A ($IC_{50} \sim 4.92 \mu M$) and U-73122 ($IC_{50} \sim 14.59 \mu M$). Cell Titre Glo assays confirmed that domperidone and carvedilol remain cytotoxic against 3D spheroids. The metabolomics-derived candidates, RSM932A and U-73122, were tested in synergy and demonstrated additive effects. These candidates also displayed minimal anti-invasive properties in a transwell invasion assay. To identify protein targets, tumour tissue samples were dissociated and subjected to 5-aminolevulinic acid-based fluorescence-activated cell sorting. Proteins from each cell population were extracted and analysed using liquid chromatography-mass spectrometry; however, the data obtained from this was unreliable. In summary, employing omics-derived targets identified potential novel therapeutic candidates against the invasive margin, thereby contributing to the expanding research field investigating this tumour region and emphasising the importance of therapeutically targeting the glioblastoma invasive margin.

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Abbreviations

5-ALA	5-aminolevulinic acid
A2M	Alpha-2-macroglobulin
ABC	ATP-binding cassette
ACTA1	Actin alpha skeletal muscle
ACTA2	Actin aortic smooth muscle
ACTB	Actin cytoplasmic 1
ACTC1	Actin alpha cardiac muscle 1
ACTG1	Actin cytoplasmic 2
ACTG2	Actin gamma-enteric smooth muscle
ANOVA	Analysis of Variance
APOL2	Apolipoprotein L2
ASCL1	Achaete-scute homolog 1
ASPSCR1	Alveolar soft part sarcoma chromosome region, candidate 1
ATP	Adenosine triphosphate
ATRX	Alpha-thalassemia/mental retardation X-linked
BBB	Blood-brain barrier
BCL-XL	B-cell lymphoma-extra-large
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CD58	Lymphocyte function-associated antigen 3
CD70	Cluster of Differentiation 70
CDV3	Carnitine deficiency-associated gene expressed in ventricle 3
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CHI3L1	Chitinase 3-like protein 1
CK	Choline kinase
CNS	Central nervous system
CORO2B	Coronin 2B
DCX	Doublecortin
DLL3	Delta-like ligand 3
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DST	Dystonin
DYSF	Dysferlin
ECM	Extracellular matrix
EDIL3	EGF-like repeats and discoidin domains 3
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
F13A1	Coagulation factor XIII A chain
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FOSL1	FOS like 1, AP-1 transcription factor subunit
GABRA1	Gamma-aminobutyric acid type A receptor alpha1 subunit
GBM	Glioblastoma
GCE	GBM core enhancing
GIN	Glioma invasive margin
GPAT	Glycerol-3-phosphate acyltransferase
GSC	Glioma stem cell
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK1	Hexokinase 1
HK2	Hexokinase 2
HK3	Hexokinase 3
HKDC1	Hexokinase domain containing 1
HSD17B12	Oestradiol 17-beta-dehydrogenase 12
IDH	Isocitrate dehydrogenase
IGHG1	Immunoglobulin heavy constant gamma 1
IGHG2	Immunoglobulin heavy constant gamma 2
IGHG3	Immunoglobulin heavy constant gamma 3
IGHG4	Immunoglobulin heavy constant gamma 4
KRT79	Keratin 79
L1CAM	L1 cell adhesion molecule
LC-MS	Liquid chromatography-mass spectrometry
LOXL1	Lysyl oxidase-like 1
MES	Mesenchymal

MET	Mesenchymal epithelial transition
MGMT	O ⁶ -methylguanine-DNA methyltransferase
MMP	Matrix metalloproteinase
MNDA	Myeloid cell nuclear differentiation antigen
MRGPRX3	Mas-related G-protein coupled receptor membrane X3
MS	Mass spectrometry
NEFL	Neurofilament light chain
NES	Neural precursor and stem cell marker
NF1	Neurofibromatosis 1
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKX2-2	NK2 homeobox 2
NPC	Neural progenitor cell
OLIG2	Oligodendrocyte transcription factor 2
OPC	Oligodendrocyte precursor cell
OS	Overall survival
PBS	Phosphate-buffered saline
PDGFR	Platelet-derived growth factor receptor
PEG	Polyethylene glycerol
PFS	Progression-free survival
PGK1	Phosphoglycerate kinase 1
PGK2	Phosphoglycerate kinase 2
PI3K	Phosphoinositide 3-kinase
PLA ₂	Phospholipase A2
PLB	Phospholipase B
PLC	Phospholipase C
PLD	Phospholipase D
POTE	Prostate, ovary, testes expressed
POTEE	POTE ankyrin domain family member E
POTEF	POTE ankyrin domain family member F
POTEI	POTE ankyrin domain family member I
POTEJ	POTE ankyrin domain family member J
PTEN	Phosphate and tensin homolog
PXK	PX domain-containing serine/threonine kinase-like protein
PZP	Pregnancy zone protein

qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RAC1	Ras-related C3 botulinum toxin substrate 1
RAC2	Ras-related C3 botulinum toxin substrate 2
RAC3	Ras-related C3 botulinum toxin substrate 3
RENBP	Renin-binding protein
RMSD	Root mean square deviation
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ROBO1	Roundabout guidance receptor 1
SEM	Standard error of the mean
SERPINE1	Serpin family E member 1
SERPINE2	Serpin family E member 2
SFN	Stratifin
SH3KBP1	SH3 domain-containing kinase-binding protein 1
SLC	Solute carrier
SLC12A5	Solute carrier family 12 member 5
SLC39A14	Solute carrier family 39 member 14
SOX	SRY-related HMG-box
SPTLC3	Serine palmitoyltransferase long chain base subunit 3
SRC	SRC proto-oncogene, non-receptor tyrosine kinase
STAT3	Signal transducer and activator of transcription 3
SUN3	Sad1 and UNC84 domain containing 3
SYT1	Synaptotagmin 1
TCF4	Transcription factor 4
TCGA	The Cancer Genome Atlas
TMZ	Temozolomide
TNF	Tumour necrosis factor
TP53	Tumour protein 53
UV	Ultraviolet
WHO	World Health Organisation
YKL40	Chitinase 3-like protein 1
YWHAB	14-3-3 protein beta
YWHAE	14-3-3 protein epsilon
YWHAH	14-3-3 protein eta

YWHAQ	14-3-3 protein theta
YWHAZ	14-3-3 protein zeta
ZNF185	Zinc finger protein 185 with LIM domain

1. Introduction

1.1 Isocitrate Dehydrogenase Glioblastoma

1.1.1 Epidemiology of Glioblastoma

Isocitrate dehydrogenase (IDH) wild-type glioblastoma (GBM) is the most common and aggressive form of brain cancer. They make up 14% of all tumours within the brain and central nervous system (CNS) and 52% of all malignant brain tumours (Price *et al.*, 2024). Due to the highly aggressive nature of this tumour, the prognosis has invariably remained very poor over several decades, with no geographical bias. The median survival is approximately 15 months with standard-of-care multimodal treatment or 3 months without treatment, with less than 5% of patients surviving 5 years after diagnosis (Thakkar *et al.*, 2014). The median age at diagnosis is 66 years (Price *et al.*, 2024). The risk of developing GBM is highest between the ages of 75 and 85, with the risk decreasing after 85 (Thakkar *et al.*, 2014). Research into the risk factors associated with GBM has been largely inconclusive. The only known environmental factor is exposure to ionising radiation (Davis, 2016). The short-term use of anti-inflammatory medication, atopic diseases, and allergies have been associated with a protective effect against GBM. There are studies investigating the effects of smoking, drug use, and mobile phones, but these are anecdotal and have provided no evidence or consistent results (Thakkar *et al.*, 2014). Several genetic conditions have been linked to an increased risk of GBM, which include Li-Fraumeni syndrome, Neurofibromatosis 1 (NF1), and Turcot syndrome (Smith *et al.*, 2024).

1.1.2 Clinical Characteristics of Glioblastoma

GBMs are classified as a grade 4 adult-type diffuse glioma by the World Health Organisation (WHO). They are highly invasive tumours as the malignant cells from the primary tumour can infiltrate the surrounding brain parenchyma, allowing the cancer to recur in localised regions after resection of the primary tumour (Lan, Li and Zhang, 2024). GBM can arise in the frontal, occipital, parietal, or temporal lobes in the supratentorial region of the brain, with the location influencing the symptoms experienced (Thakkar *et al.*, 2014; Louis *et al.*, 2016). Currently, brain tumours are initially diagnosed through magnetic resonance imaging, followed by a definitive

diagnosis whereby classification of the tumour is made through histopathological examination of a biopsy sample, increasingly complemented by genome sequencing to determine the molecular subtype (Czarnywojtek *et al.*, 2023).

There is much debate surrounding the cell-of-origin for GBM, with studies implicating numerous cell types. GBM initiation has been shown to arise from neural stem cells, glial precursor cells, oligodendrocyte precursor cells and astrocytes that have gained somatic mutations (Paw *et al.*, 2015; Kim, Park and Lee, 2021; Li, Feng and Lu, 2023). An active area of interest is a population of cells within the tumour known as glioma stem cells (GSCs), which are self-renewing, undifferentiated cells (Paw *et al.*, 2015). GSCs are involved in the initiation, progression, and recurrence of tumours. They may also contribute to treatment resistance and invasion into the surrounding brain (Sloan *et al.*, 2024). While this population has been linked to disease progression, there is no definitive evidence that they are the cell-of-origin, and the definitive cell type is yet to be conclusively determined.

1.1.3 Classification of Glioblastoma

In 2016, the WHO classification of brain and CNS tumours established molecular parameters required for diagnosis alongside the existing histopathological features, allowing for more narrowly defined tumour types and subtypes (Louis *et al.*, 2016). This has allowed for more accurate diagnosis and better predictions regarding prognosis and treatment response for the individual patient. More recently, the 2021 WHO classification of brain and CNS tumours was published, which builds on the 2016 classification. It incorporates additional molecular biological markers associated with the required or desired molecular changes (Louis *et al.*, 2021). This has allowed for more accurate classifications of brain and CNS tumours, providing clinicians with a better understanding of the individual's tumour, which can inform better treatment decisions.

Primary GBMs typically arise from an unknown malignant precursor (i.e., arises *de novo*). They primarily occur in older patients and make up the majority of cases. Secondary GBMs arise from a known precursor, typically a grade 2 astrocytoma, and occur primarily in younger patients (Louis *et al.*, 2016). Primary GBM tumours can be

further classified based on their molecular characteristics and markers. In 2006, Phillips *et al.* classified GBMs into 3 distinct groups based on their molecular signatures: proneural, proliferative and mesenchymal (Phillips *et al.*, 2006). In 2010, Verhaak *et al.* identified four subtypes: classical, proneural, neural and mesenchymal, displayed in Table 1.1 (Verhaak *et al.*, 2010).

Table 1.1: Molecular characteristics of GBM subtypes according to Verhaak *et al.* (2010). EGFR = epidermal growth factor receptor. TP53 = tumour protein 53. CDKN2A = cyclin-dependent kinase inhibitor 2A. NES = neural precursor and stem cell marker. PDGFR = platelet-derived growth factor receptor. PTEN = phosphate and tensin homolog. MET = mesenchymal epithelial transition. NF-kB = nuclear factor kappa-light-chain-enhancer of activated B cells. TNF = tumour necrosis factor.

Classical	Proneural	Neural	Mesenchymal
- Amplification of chromosome 7 and loss of chromosome 10 - EGFR amplification - Lack of <i>TP53</i> mutations - <i>CDKN2A</i> deletion - Overexpression of Notch and Sonic Hedgehog signalling pathways - Overexpression of NES	- <i>TP53</i> mutations - Point mutations in IDH1 - PDGFR amplification - Some amplification of chromosome 7 and loss of chromosome 10 - High oligodendrocytic gene expression: <i>PDGFRA</i>, <i>NKX2-2</i>, <i>OLIG2</i> - Proneural development gene expression: <i>SOX</i>, <i>DCX</i>, <i>DLL3</i>, <i>ASCL1</i>, <i>TCF4</i>	- Expression of neuronal markers: <i>NEFL</i>, <i>GABRA1</i>, <i>SYT1</i>, <i>SLC12A5</i> - Normal cells present in pathology slides	- PTEN mutations - Decreased NF1 expression - Expression of mesenchymal CHI3L1/YKL40 and MET markers - Overexpression of genes in NF-kB pathway and TNF superfamily

However, it was later suggested that for the Verhaak *et al.* neural classification, the sample may have been contaminated by healthy brain tissue, which explains why no gene abnormalities were observed (Wang *et al.*, 2017).

The classification of GBM into subtypes can help predict prognosis and the patient's response to treatment, potentially aiding treatment decisions. For example, proneural tumours conferred the best relative prognosis of all the subtypes and often affected younger individuals (Verhaak *et al.*, 2010). Verhaak *et al.* also showed that overall survival (OS) increased in classical and mesenchymal tumours when aggressive treatment was used, where this treatment did not affect survival in proneural tumours (Verhaak *et al.*, 2010).

1.2 Current Standard of Care for Glioblastoma

Despite how debilitating this tumour is, GBM remains incurable and current treatments aim to increase OS and improve a patient's quality of life. The current standard of care is the Stupp protocol which involves maximal safe tumour resection, followed by adjuvant radiotherapy with concomitant temozolomide (TMZ) chemotherapy. The addition of TMZ to the Stupp protocol was clinically adopted in 2005 when Stupp *et al.* found that the addition of TMZ therapy increased the median survival by 2.5 months and the 2-year survival rate by 16% (Stupp *et al.*, 2005).

It has been shown that a greater extent of tumour resection and lower residual tumour volume correlate with an increased overall and progression-free survival (PFS) (Gerritsen *et al.*, 2023). However, due to the invasive nature of this tumour, it is impossible to surgically remove all the malignant cells. 5-aminolevulinic acid (5-ALA) is now used during surgery as a neuronavigational tool to help the surgeon achieve the greatest tumour resection possible. 5-ALA accumulates within the mitochondria of GBM cells and is visible as pink fluorescence under ultraviolet light that can be observed during surgery (Obrador *et al.*, 2024). Despite the amount of research into GBM and potential therapeutics in the last 20 years, the standard of care in the United Kingdom has not changed since the introduction of the Stupp protocol in 2005.

1.3 Challenges in Treating Glioblastoma

1.3.1 The Blood-Brain Barrier

A major challenge in targeting therapeutics to the brain across a range of neurological conditions is the blood-brain barrier (BBB). This maintains the physiological state of the CNS. The BBB is a tightly regulated, semi-permeable membrane that protects the brain from toxic substances and pathogens (Mathew-Schmitt *et al.*, 2024). While this barrier protects the brain from neurotoxic agents to maintain homeostasis, it also impairs CNS entry of many therapeutic drugs. Indeed, the BBB keeps out large molecular drugs and over 98% of small molecular drugs (Wu *et al.*, 2023). This makes it extremely difficult to target GBM cells that have spread throughout the brain using a drug that is intravenously administered or ingested (i.e., systemic administration). Both the BBB and GBM cells express a range of efflux transporters such as multidrug resistance proteins, P-glycoproteins, breast cancer resistance proteins, and organic anion transporters (Obrador *et al.*, 2024). These transporters pump the drug out of the cells or brain before it can have any therapeutic effect. Drugs that are effective *in vitro* may not be efficacious *in vivo* due to their inability to penetrate the BBB or reach a therapeutic concentration within the brain. To combat this, a higher concentration of the drug would be needed. However, this can cause adverse dose-limiting effects due to an increased plasma drug concentration and widespread distribution, which could trigger potentially life-threatening toxicity affecting not only the brain but the whole body (Duan *et al.*, 2024).

1.3.2 Glioblastoma Heterogeneity

Another major challenge when treating GBM is high inter- and intra-patient heterogeneity (Kim, Park and Lee, 2021; Sloan *et al.*, 2024). Within biology, heterogeneity refers to differences or variations across biological entities such as cells, tissues, or individuals within a population. GBM displays high inter-patient heterogeneity, making it harder to treat because no two tumours are the same. Verhaak *et al.* identified four subgroups, each with different molecular characteristics, by which an individual's tumour could be classified (Verhaak *et al.*, 2010). Different tumours will display different molecular characteristics and driver mutations, making it difficult to identify a universal treatment for this disease. For example, some patients

display methylated O⁶-methylguanine-DNA methyltransferase (MGMT), a deoxyribonucleic acid (DNA) damage repair enzyme which can protect tumours against alkylating chemotherapy agents such as TMZ. When the MGMT promoter is methylated, the gene is silenced and cells no longer produce MGMT, so DNA damage is no longer repaired (Esteller *et al.*, 2000; Hegi *et al.*, 2004). Methylated MGMT is associated with better survival outcomes after treatment with TMZ and can be used to predict which patients will respond well (Hegi *et al.*, 2004; Hegi *et al.*, 2005). GBM's highly heterogeneous nature thus makes the development of novel therapeutics extremely challenging. Further confounding treatment efforts, GBM also displays intratumoural heterogeneity. Brennan *et al.* showed that there is not one single oncogene that drives GBM tumour progression, but many. They identified various driver mutations in EGFR, PDGFRA, MET, phosphoinositide 3-kinase (PI3K) and TP53 (Brennan *et al.*, 2013). Using the Verhaak classification, Sottoriva *et al.* showed that samples taken from the same tumour, but different regions, can be classified as different molecular subtypes, with some patients displaying two or more subtypes (Verhaak *et al.*, 2010; Sottoriva *et al.*, 2013). Classifying GBMs into subtypes can help predict prognosis but using it for informing treatment can lead to missing smaller subpopulations of malignant cells which may survive and continue to drive tumour progression. Netfai *et al.* showed that GBM cells within a tumour can be mapped to one of four different cellular states: oligodendrocyte precursor cell (OPC) like, astrocyte like, neural progenitor cell (NPC) like and mesenchymal (MES) like. Different subtypes displayed differences, with tumours enriched with astrocyte-like cells expressing upregulated EGFR, OPC- and NPC-like cells expressing amplified PDGFRA and cyclin-dependent kinase 4, and MES-like cells expressing NF1 alterations (Netfai *et al.*, 2019). Heterogeneity is seen at the cellular level which poses a problem as treatments may only be effective against certain sub-populations, allowing surviving cells to continue proliferating. This surviving population can select for a treatment-resistant population, making it harder to treat. Our laboratory has recently shown that cells from the core, rim, unsorted invasive margin, and the 5-ALA positive and negative sorted regions in the invasive margin display distinct transcriptomic differences (Andrieux *et al.*, 2023). This spatial heterogeneity can mean that treatments may only target certain regions of the tumour and will not have an effect on other regions. Due to this, it is important to focus on the most clinically

relevant region of GBM, which is the invasive margin, as this drives recurrence, to increase the chances of finding a successful therapeutic.

1.4 The Invasive Margin

1.4.1 Tumour Recurrence

Tumour recurrence is virtually inevitable and typically occurs six to nine months after resection of the original tumour (Mallick *et al.*, 2016). GBM cells are highly malignant and invasive, thereby infiltrating the surrounding healthy brain tissue before treatment can be administered for the primary tumour (Lan, Li and Zhang, 2024). These infiltrative disease cells rapidly divide, leading to tumour recurrence, typically occurring within two to three centimetres from the location of the primary tumour (Obrador *et al.*, 2024). The area around the tumour harbouring residual infiltrative cells is known as the 'invasive margin' (Andrieux *et al.*, 2023).

To migrate away from the primary tumour through the brain tissue, GBM cells can change their shape and volume so they can fit in small spaces in the brain (Paw *et al.*, 2015). These cells develop a leading edge in response to chemical, mechanical and electrical cues, where actin polymerisation occurs. This creates membrane protrusions that the cell uses to attach to the extracellular matrix (ECM) to promote motility and tissue invasion (Paw *et al.*, 2015; Mair, Ames and Li, 2018). The ECM is an impermeable barrier that cells are usually unable to pass through. To overcome this, GBM cells display an increased expression of matrix metalloproteinases (MMPs). These are proteolytic enzymes released from the cell that work synergistically to degrade and remodel the ECM, allowing the malignant cells to pass through and spread to neighbouring brain regions (Ahir, Engelhard and Lakka, 2020). MMP-2 and MMP-9 expression is upregulated in GBM and has been linked to cell invasiveness, tumour progression, and poorer patient outcomes (Du *et al.*, 2008; Xue *et al.*, 2017). Currently, therapeutics do not effectively target and eradicate these invasive cells which remain in the brain after treatment and lead to the growth of a recurrent tumour.

1.4.2 Treating Recurrent Glioblastoma

These migratory and invasive cells pose a huge problem when trying to treat GBM. Surgical resection is not curative as it only removes the bulk tumour. It does not remove all the malignant cells that have infiltrated into the surrounding brain tissue, even with the use of 5-ALA. These remaining cells can proliferate, leading to disease progression and the formation of a recurrent tumour which is extremely difficult to treat. Once the tumour reoccurs, there is no established standard of care, and most patients are not eligible for further treatment. Treatment for recurrent GBM is limited to surgery if safe to re-operate (i.e. if the recurrent tumour is in a non-eloquent brain region), re-irradiation if the tumour is far from the location of the primary tumour, and chemotherapy. These treatments aim to manage symptoms and improve the quality of life for the patient but only offer a slight increase in OS (Mallick *et al.*, 2016). However, recurrent GBM often displays treatment resistance to both radiotherapy and TMZ chemotherapy (Shergalis *et al.*, 2018). There is evidence that exposure of GBM cells to ionising radiation can trigger a switch to a mesenchymal state through the NF- κ B pathway (Chen *et al.*, 2022). This switch aids cellular migration and promotes the development of an invasive phenotype within the invasive margin, for which no effective treatment exists.

Another contributor to treatment resistance is GSCs. These cells rapidly renew and can differentiate into different cellular populations, selecting for treatment-resistant cell types (Obrador *et al.*, 2024). GSCs can exit the cell cycle and become quiescent, entering a temporary stage of growth arrest, allowing them to avoid therapeutics that target rapidly dividing cells. However, they can re-enter the cell cycle if stimulated by an external factor or stimulus such as injury (Wang *et al.*, 2025). This population of cells often displays amplification of the DNA damage repair response (Bao *et al.*, 2006). They also exhibit a downregulation of proapoptotic genes and an upregulation of antiapoptotic genes, making it difficult to trigger cell death pathways with therapeutics (Sloan *et al.*, 2024). While tumour recurrence is inevitable, there is no effective treatment for it.

1.4.3 Glioblastoma Tumour Regions

GBM tumours consist of three distinct regions (Figure 1.1). The necrotic tumour core and the proliferative rim make up the bulk tumour, which is typically removed during surgery. The invasive margin surrounding this contains both malignant GBM cells and somatic brain cells, including neurons, astrocytes, microglia, and infiltrating immune cells (Read *et al.*, 2024).

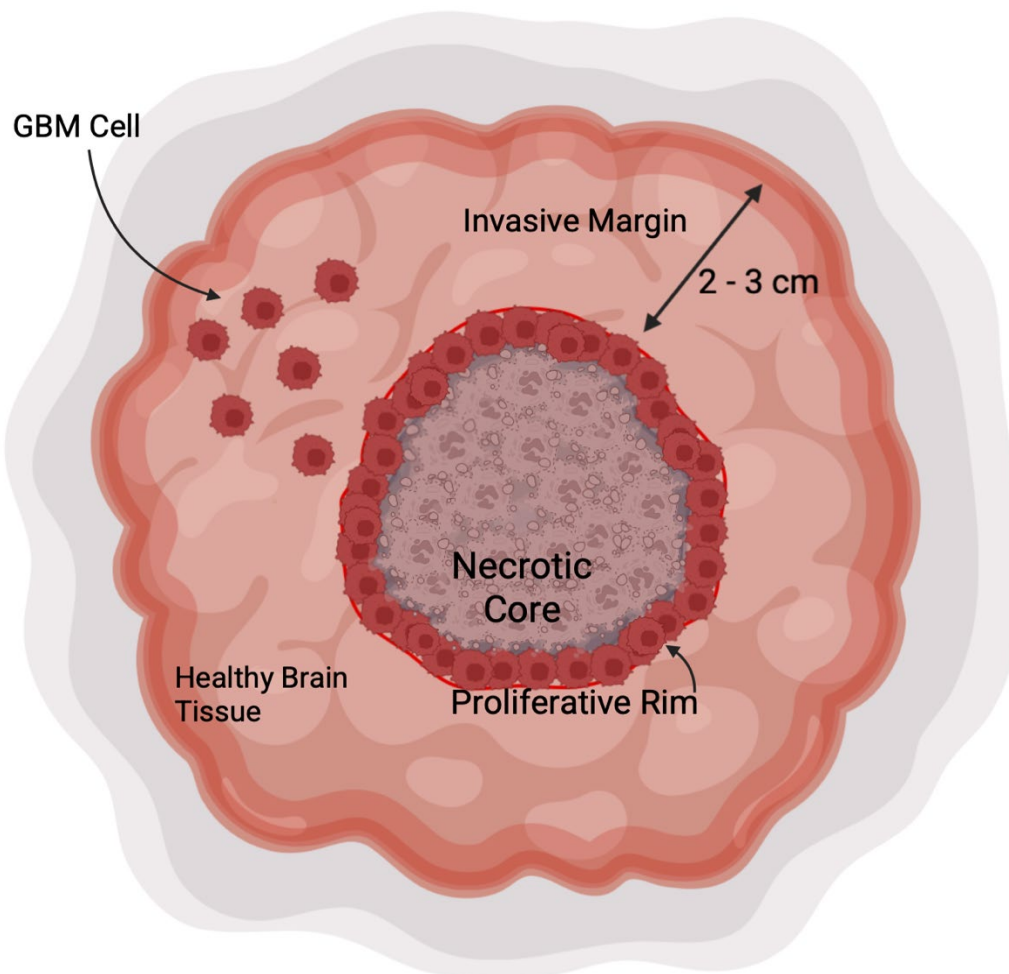


Figure 1.1: The tumour regions within glioblastoma. Glioblastomas have three distinct tumour regions: the necrotic tumour core, surrounded by a rim of rapidly proliferating GBM cells and the invasive margin. The invasive margin is the area around the brain tumour where GBM cells have migrated away from the primary tumour into the healthy brain tissue. This migration typically happens within 2 – 3 centimetres of the original tumour (Smith *et al.*, 2017). Created in BioRender.

The use of 5-ALA during surgery has allowed more precise identification of the invasive margin that surrounds the bulk tumour. The routine use of 5-ALA during surgery helps surgeons achieve the safest maximal resection possible as it allows them to distinguish GBM cells from healthy tissue. 5-ALA is taken up by both

cancerous and somatic cells within the brain and metabolised by the heme biosynthetic pathway (Figure 1.2). In GBM cells, there are reduced levels of the enzyme ferrochelatase, meaning that protoporphyrin IX is not converted into heme as it is in healthy neural cells, but instead accumulates in the mitochondria, emitting pink fluorescence under an ultraviolet light (Smith *et al.*, 2017; Andrieux *et al.*, 2023). Even though 5-ALA allows for a greater extent of resection, complete maximal resection of the cancer is extremely difficult and unattainable.

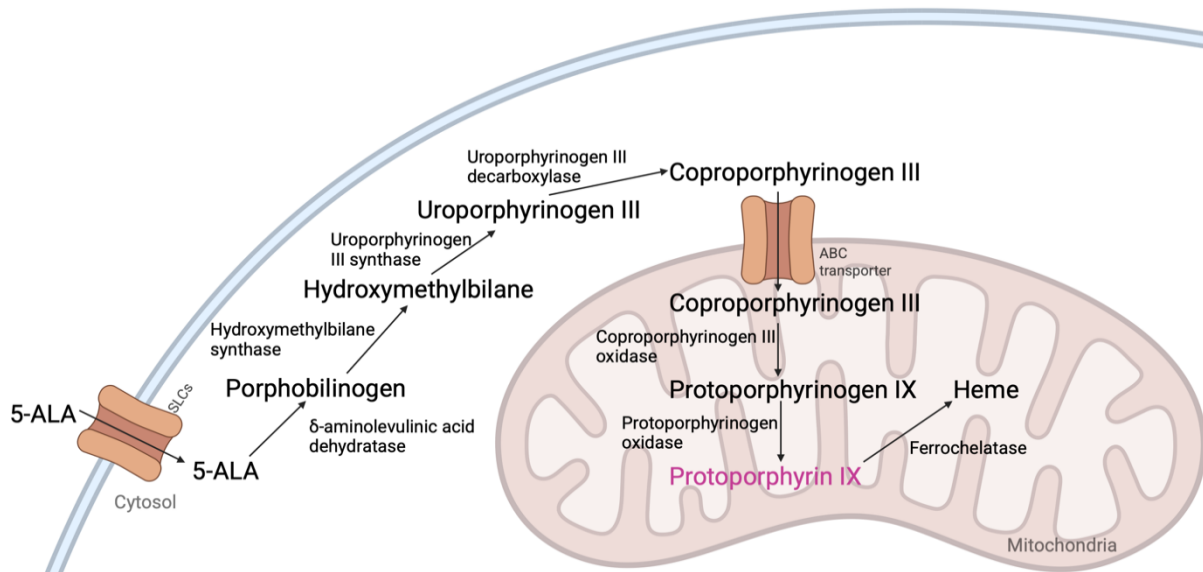


Figure 1.2: 5-ALA is metabolised by the heme biosynthetic pathway once it is taken up by the cell. 5-ALA produces coproporphyrinogen III in the cytosol which is then transported inside the mitochondria and further metabolised to produce protoporphyrin IX. Protoporphyrin IX accumulates within the mitochondria and fluoresces pink under an ultraviolet light in GBM cells (Smith *et al.*, 2017; Andrieux *et al.*, 2023). SLCs = Solute carriers. ABC = ATP-binding cassette. Created in BioRender.

The necrotic tumour core does not fluoresce, but the surrounding rim brightly fluoresces. This fluorescence will continue into the invasive margin but will decrease as the number of invasive cells present decreases, and the distance from the tumour core increases (Andrieux *et al.*, 2023). This has allowed surgeons to visualise the extent of invasion and remove more of the tumour than previously possible.

1.4.4 Glioblastoma Regions Display Distinct Molecular Differences

GBM exhibits a high degree of intra-tumour heterogeneity. It has been shown that cells within the invasive margin exhibit distinct molecular differences relative to the primary tumour core from which they originated (Smith *et al.*, 2017). Our laboratory found that only 9.1% of all differentially expressed genes were conserved across the tumour core, peripheral rim, and invasive margin, with the greatest difference being between the

invasive margin and tumour core. The 5-ALA positive cells in the invasive margin exhibited an upregulation of genes involved in migration, invasion and ECM organisation and reduced levels of proliferation (Smith *et al.*, 2020). Using the subtypes described by Verhaak *et al.* in 2010, the core and rim populations show enrichment for the classical and proneural subtype, whereas the invasive margin shows enrichment for the mesenchymal subtype (Verhaak *et al.*, 2010; Andrieux *et al.*, 2023). Further research within our laboratory by Andrieux *et al.* showed that the tumour core is enriched with pro-proliferative pathways that are absent within the invasive margin. They also identified a significant enrichment of genes associated with cell mobility and invasion, immune and inflammatory response, and evading cell death, in the 5-ALA positive invasive margin cells. This 5-ALA population has been linked to tumour recurrence through *in vivo* work and is associated with poorer survival outcomes (Andrieux *et al.*, 2023). These studies show that the invasive margin displays distinct molecular differences from the tumour core. As the invasive margin is in proximity to residual disease, which cannot be safely resected during surgery, targeting and eradicating this population could prevent or delay recurrence and improve patient outcomes. These findings underscore the need for a shift in focus from the tumour core to the invasive margin region in GBM research, as it may inform more clinically accurate effective strategies for treating GBM.

The majority of the invasive margin is comprised of 5-ALA negative cells, which are presumed to be normal, non-cancerous cells, albeit residing within the tumour microenvironment. Investigation into the molecular differences between the 5-ALA positive and 5-ALA negative populations in the invasive margin could identify selective drug targets for these malignant cells, which are absent or expressed at much lower levels in non-malignant cells (i.e., offer a therapeutic window). A previous study conducted by our laboratory identified potential biomarkers and found significant upregulation of 78 genes and downregulation of 102 genes in the 5-ALA positive population compared to the 5-ALA negative population (Smith *et al.*, 2020). This is the first study to our knowledge to investigate differences between the two populations and could offer a way to selectively target the GBM cells in the invasive margin whilst minimising toxicity to the surrounding healthy brain tissue.

Previous GBM research has almost exclusively focused on samples reflecting the tumour core which is removed during surgery. As different tumour regions are molecularly variable, research into molecular markers from the invasive margin will provide a better reflection of the residual disease remaining after surgery. As this residual disease is directly linked to tumour recurrence, targeting this population could prevent tumour recurrence, dramatically improving patient outcomes (Smith *et al.*, 2020). Investigation into this 5-ALA positive population could identify specific biomarkers that could allow us to better understand the invasive nature of these cells and act as superior targets for novel or repurposed drugs.

1.5 Identification of Novel Drug Candidates for the Invasive Margin

Before novel drug candidates can be developed for any particular condition, a rational drug target must first be identified. Drug targets are biological molecules, such as proteins, that are commonly dysregulated in diseases through mutation (genetic or epigenetic), increased or decreased expression, or over- or under-activity. Therapeutics can be developed that directly interact with dysregulated molecules to counter their effects and provide a therapeutic benefit. While multiple targets have been identified with clinical relevance for GBM, such as the EGFR, the PI3K pathway, TP53 and the retinoblastoma pathway, targeting these has not resulted in any success in the clinic. Indeed, no molecular targeted therapy has reported efficacy in phase III clinical trials for GBM (Le Rhun *et al.*, 2019). Such molecular targets have previously been identified via genome sequencing predicated upon cells derived from the tumour core instead of the invasive margin, which could explain why they failed to produce a therapeutic benefit. Targets that have been identified using tissue or cells from the bulk tumour may not be present in the invasive margin cells, due to intra-tumour heterogeneity. As invasive margin cells are likely associated with recurrence, there is a need to identify precise molecular targets within this population so that therapeutics can be specifically targeted to these cells. Using targets from this population will increase the chances of success in finding a drug candidate which can prevent or delay recurrence and increase survival rates for the patient. Common approaches to identifying potential drug targets involve the use of omics technologies and molecular docking.

1.5.1 The Use of Omics Technologies in Drug Development

Omics refers to the ability to simultaneously analyse thousands of biological molecules to investigate the entire makeup of a given biological system. It can reveal the structure and function of these molecules, how they influence each other, and allow the intricate molecular mechanisms underlying diseases to be uncovered (Veenstra, 2021; Dai and Shen, 2022). Omics technologies allow for a high-throughput analysis of biological molecules, allowing the identification and processing of huge databases in relatively short amounts of time. Numerous databases have been constructed to store this data to make it openly accessible. Some examples of these are The Cancer Genome Atlas (TCGA), Ivy Glioblastoma Database, and Glioma Bio Discovery Portal. The “big four omics” refers to genomics, transcriptomics, proteomics, and metabolomics (Dai and Shen, 2022). The use of these techniques allows for rapid identification of biomarkers which can inform disease progression and appropriate treatment methods. Identification of biomarkers can provide clinically relevant drug targets against which novel therapeutics for the disease can be developed (Figure 1.3).

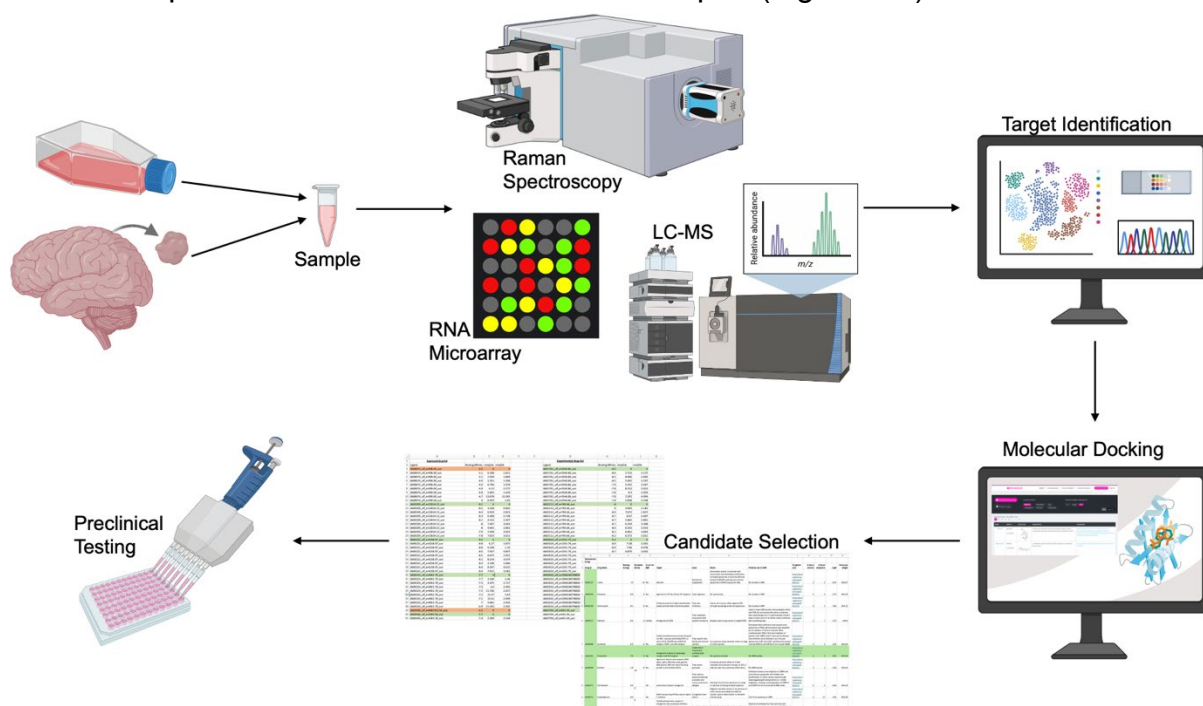


Figure 1.3: The workflow of identifying novel therapeutics with the use of omics approaches and molecular docking. Cell lines or patient samples are collected and processed to extract biological molecules such as proteins, ribonucleic acid (RNA) or metabolites, which are analysed using omics approaches. When the samples are being run, they generate huge lists of all the molecules detected in the sample, which can be used to identify potential drug targets. The identified drug targets can be used in molecular docking, where databases of drugs are docked against the target to produce a list of candidate drugs that could bind. These lists are then narrowed down to produce the final candidates before being taken into *in vitro* testing. LC-MS = liquid chromatography-mass spectrometry. Created using BioRender.

GBM is a highly complex and heterogeneous disease at both the cellular and molecular levels, making it difficult to treat. This leads to the reasoning that to understand this disease and better treat it, methods are required that can capture this heterogeneity in a way that it can be analysed. Through the use of omics, the entire makeup of a cell can be analysed, providing a wealth of information that would previously have been impossible to gain in such a short time (Dai and Shen, 2022). It has allowed researchers to identify molecular differences between tumour tissue and healthy tissue, and between different patients.

Transcriptomics analyses all RNA transcripts present in a cell, tissue, or organism (Lowe *et al.*, 2017). It provides information regarding gene expression within the sample and can study how it changes within disease states to aid the identification of pathways that play a role in disease (Lowe *et al.*, 2017; Magro, Venezia and Rita Balistreri, 2024). As gene expression is dynamic, alterations of internal or external stimuli, time points, and even cell development stages can alter the expression level of a specific gene (Dai and Shen, 2022). This allows genes to be identified that are activated at a particular time point, or after exposure to a stimulus, and the relative level of expression of these genes. Transcriptomics uses sequencing-based techniques such as microarray analysis and RNA sequencing (RNA-seq) (Dai and Shen, 2022). RNA-seq can capture and quantify all RNA transcripts (both coding and non-coding) present using high-throughput sequencing combined with computational methods (Lowe *et al.*, 2017).

Previous research in our laboratory conducted by Smith *et al.* (2020) showed upregulation of 78 genes and downregulation of 102 genes in the 5-ALA positive cells compared to the 5-ALA negative cells in the GBM invasive margin. Through transcriptional analysis, *SERPINE1* was identified as a potential drug target which is highly expressed in 5-ALA positive cells but not in the surrounding healthy brain tissue. Through knockdown and pharmacological inhibition of this gene, the invasiveness of GBM invasive margin cells was reduced (Smith *et al.*, 2020). Further studies by Andrieux *et al.* (2023) found that the 5-ALA positive cells in the invasive margin displayed a mesenchymal subtype through transcriptomic profiling. The study found that glycolysis-related genes are upregulated in these cells, and inflammatory wound response and tissue repair transcriptional signatures were enriched (Andrieux *et al.*,

2023). This shows the potential of using transcriptomics to identify genes associated with disease progression, which represent potential therapeutic targets.

Proteomics has enabled the identification and analysis of all proteins present within a cell, tissue, or organism, which is referred to as the proteome (Sun and Hu, 2016). It can reveal the identity, expression level, and function of thousands of proteins within a given biological sample (Kwon *et al.*, 2021). Dysregulated protein expression can trigger aberrant signalling pathways within GBM cells, allowing them to rapidly proliferate, evade cell death, metastasise, and become treatment resistant. An example of this is the EGFR protein involved in angiogenesis, migration, and metastatic spread of the tumour. EGFR is amplified in some GBMs, meaning this signalling pathway is overactive, promoting tumour growth and cancer progression (Ross *et al.*, 2017). Investigation into differential protein expression and the associated cellular behaviour in GBM cells could uncover novel drug targets that restore normal cellular behaviour. Inhibiting or activating specific signalling pathways may reactivate dormant pathways, for example, cell death pathways, or block overactive pathways. Proteomics utilises mass spectrometry (MS) methods such as liquid chromatography-MS (LC-MS). Proteins are extracted and digested to produce peptides which are separated by liquid chromatography. These peptides are ionised, and the mass spectrometer measures the mass-to-charge ratio of the peptide and the signal intensity. This generates a spectrum of peaks plotting the mass to charge ratio against retention time. These peaks can be matched to known proteins in databases, and the abundance of each identified protein in the cell can be determined, generating a list of every protein detected within a biological sample (Shuken, 2023).

A study conducted by Ross *et al.* (2017) used samples taken from the GBM invasive margin and found that proteins involved in pro-survival processes and anti-apoptotic pathways, such as SRC, PTEN and BCL-XL were upregulated, potentially contributing to treatment resistance (Ross *et al.*, 2017). Previous research in our laboratory by Kocon *et al.* (2025) investigated protein expression within the 5-ALA derived invasive margin and identified cAMP-response element-binding protein and protein kinase B as upregulated in this tumour region (Kocon *et al.*, 2025). However, these samples were unsorted by FACS, and therefore, these proteins could also be present in non-cancerous cells. While there have been numerous studies investigating protein

expression within GBM, there has yet to be any clinical benefit. The proteome of the invasive margin and residual disease is poorly understood, and many previous protein targets that have been identified are from the tumour core. Identifying proteins in GBM cells from the invasive margin could provide a means of finding or developing therapeutics that can selectively kill these invasive cells to prevent recurrence of this tumour.

Metabolomics analyses the complete set of metabolites and their interactions within a biological system, which is referred to as the metabolome (Magro, Venezia and Rita Balistreri, 2024). Metabolites are downstream of the genome, transcriptome and proteome and drive various cellular processes such as energy production, signal transduction, and homeostasis. As metabolites are downstream and highly dynamic, slight perturbations in the gene, transcript or protein level can influence the metabolome (Taunk *et al.*, 2024). Oncometabolites are endogenous metabolites that are involved in tumour growth and metastasis and can accumulate within cancer cells to produce downstream effects (Kumar and Misra, 2019; Taunk *et al.*, 2024). The first identified oncometabolite was 2-hydroxyglutarate in IDH1 mutant gliomas (Dang *et al.*, 2009). Similar to proteomics, metabolomics involves the use of MS-based methods such as LC-MS and gas chromatography MS, but also involves spectroscopy techniques such as Raman and nuclear magnetic resonance (Taunk *et al.*, 2024).

Metabolomics can take two forms: targeted and non-targeted forms. Targeted metabolomics provides information for a specific set of metabolites, whereas non-targeted metabolomics characterises as many metabolites as possible in an unbiased manner and measures the abundance of these (Dai and Shen, 2022; Taunk *et al.*, 2024). Recent studies conducted by Wood *et al.* (2025) in our laboratory used metabolomics to investigate intra-tumour heterogeneity of GBM. The study showed that the invasive margin exhibited a distinct metabolome compared to the tumour core, and several of the significantly altered metabolites were associated with purine and pyrimidine metabolism (Wood *et al.*, 2025). Identification of dysregulated metabolites such as lipids, amino acids or carbohydrates that influence GBM progression would provide vital targets downstream of genes and proteins for therapeutic targeting.

1.5.2 Integration of Omics Methods with *in Silico* Molecular Docking

Omics methods can identify drug targets which can be used in high-throughput virtual screening to identify potential drug candidates. The use of *in silico* molecular docking enables the screening of millions of compounds in a relatively short amount of time. Molecular docking can predict the conformation of a ligand within a binding pocket of a target protein, and rank these ligands based on the predicted binding affinity of the ligand-protein complex (Ferreira *et al.*, 2015; Shaker *et al.*, 2021). Molecular docking can use 'ligand-based screening,' which searches for new compounds that share similarities with known active compounds that bind, or 'structure-based screening,' which explores the affinity of a compound against a target protein (Gan *et al.*, 2022). Structure-based methods use the 3D structure of the protein and dock compounds from databases such as PubChem, DrugBank, and ChemSpider against selected binding sites (Ferreira *et al.*, 2015; Pinzi and Rastelli, 2019). These compounds are then filtered, and additional molecular dynamic simulations can be conducted to further refine the compounds, allowing drug candidates to be selected for experimental validation (Shaker *et al.*, 2021). DrugRep is a molecular docking software that uses approved and experimental drug libraries from DrugBank to perform molecular docking against a target protein to produce a list of compounds that could bind (Gan *et al.*, 2022). Successful uses of molecular docking for drug repurposing have identified erlotinib for pancreatic cancer, imatinib for chronic myeloid leukaemia and lapatinib for breast cancer (Shaker *et al.*, 2021).

1.5.3 Using Repurposed Drugs for Glioblastoma

The use of molecular docking against disease target proteins allows for the identification of both clinically approved and experimental drugs that may confer a therapeutic benefit. Drug repurposing involves the identification of new applications for an already clinically approved drug. Traditional drug discovery involves the identification of a target, preclinical testing of the drug using *in vitro* and *in vivo* models, before progression to clinical trials. Preclinical testing determines the efficacy, pharmacokinetics, pharmacodynamics, toxicity profiles, metabolic profiles, mechanism of action, and the different target interactions of the drug. This process can take 10 to 15 years and can cost up to \$2 billion, with few potential candidates making it into clinical trials (Turabi *et al.*, 2022). Using repurposed drugs reduces this

time and cost as there is already prior knowledge of the drug's safety, dosage and toxicity, and therefore many of these preclinical tests do not need to be repeated. When repurposed drugs for a new indication are identified, they are tested preclinically using *in vitro* and *in vivo* disease models to fill any knowledge gaps not available from prior tests. The drug candidates can then progress faster into clinical trials, with some repurposed drugs circumventing phase I clinical trials if there is already adequate data available for dosing and side effects (Xia *et al.*, 2024). Using repurposed drugs for GBM will therefore speed up the process of testing them in preclinical models, potentially making them available to patients much earlier.

1.6 Rationale, Hypotheses and Objectives

GBM is an aggressive and incurable disease leading to very short survival times due to tumour recurrence. The current standard of care in the United Kingdom has not changed since 2005, despite the extensive amount of research that has been conducted. There is a need for new, effective therapeutics that will mean the end of GBM being seen as an incurable condition. For many years, research into GBM has been conducted using cells or tissue samples obtained from the tumour core that is typically removed during surgery. However, the use of 5-ALA has revealed the extent of GBM invasiveness and led to a better molecular understanding of the invasive margin region. This region is representative of the residual disease left behind after surgery, which has been linked to recurrence (Smith *et al.*, 2020). Numerous studies from our laboratory have investigated the molecular differences between the invasive margin region and the tumour core. They have shown that there are distinct molecular differences which may explain why potential therapeutics have historically failed to demonstrate efficacy in clinical trials (Smith *et al.*, 2017, 2020; Andrieux *et al.*, 2023; Kocon *et al.*, 2025; Wood *et al.*, 2025). As the invasive margin cellular population in GBM is linked to disease recurrence, current research should focus on this region. This population can provide viable drug targets and lead to the development of successful therapeutics.

This project aims to assess the cytotoxicity of candidate drugs identified through omics approaches against cells derived from the GBM invasive margin. Glioma INvasive Margin (GIN) cell lines are primary cell lines derived from the 5-ALA fluorescent

invasive margin in GBM. By focusing on this critical region, this project seeks to identify novel drug candidates that can prevent tumour recurrence and improve patient outcomes. The project builds upon previous research within the group that has employed transcriptomics, proteomics and metabolomics to identify potential drug targets or dysregulated pathways.

Hypothesis 1:

- Repurposed therapeutics targeting previously identified drug targets will disrupt tumour cell growth and trigger cell death in the invasive margin population.

Objectives:

- Evaluate candidate drugs based on molecular docking screening and their properties to identify successful candidates to progress to *in vitro* testing.
- Assess the cytotoxicity of these drugs in a panel of GIN cell lines.

Unpublished research within the group has identified proteomic, transcriptomic and metabolomic drug targets within the GBM invasive margin. Recently, drug targets in the form of upregulated proteins within GIN cell lines, that are absent in human astrocytes, have been identified. These proteins represent ideal therapeutic targets for this disease. However, the identification of therapeutic protein targets in GBM 5-ALA positive tissue samples remains unexplored. This project therefore aims to identify potential drug targets in the form of ubiquitous, differentially expressed proteins in GBM invasive margin tissue samples.

Hypothesis 2:

- Differentially expressed proteins in the 5-ALA positive cell population will provide potential drug targets and should overlap with those previously identified in GIN cell lines.

Objectives:

- Extract total, membrane and cytosolic proteins from 5-ALA positive and 5-ALA negative sorted cell populations.
- Analyse proteomic profiles produced using LC-MS to identify ubiquitous proteins in the 5-ALA positive population which represent drug targets.

2. Methods

2.1 Cell Lines

Experiments used primary cell lines derived from human brain tumour tissue that were obtained from patients at Queen's Medical Centre, Nottingham. Use of these cell lines was approved by the National Health Service Research and Ethics Committee East Midlands (Ref: 11/EM/0076), and such research is subject to audits by the Human Tissue Authority. The GIN8 cell line was isolated from the medial front invasive margin of a primary wild-type IDH glioblastoma (54-year-old female, 0% MGMT promoter methylation, intact alpha-thalassemia/mental retardation X-linked (ATRX), 90% resection with Gliadel wafers, treatment of 60 Gy radiotherapy concurrent and adjuvant temozolomide, died 5 months after surgery). The GIN28 cell line was isolated from the fluorescent 5-ALA positive invasive margin of a primary wild-type IDH glioblastoma (71-year-old male, 0% MGMT promoter methylation, intact ATRX, 99% resection, no adjuvant therapy, died 3 months after surgery). The GIN31 cell line was isolated from the fluorescent 5-ALA positive invasive margin of a primary wild-type IDH glioblastoma (0% MGMT promoter methylation, intact ATRX, 100% resection, treatment 60 Gy radiotherapy, concurrent and adjuvant temozolomide, died 16.1 months after surgery). These cell lines were obtained using previously described methods (Smith *et al.*, 2017, 2020).

2.2 Generation of Previous Data Required for This Project

2.2.1 Transcriptomic-Based Drug Selection

The following work was carried out by Maria Shah at the University of Nottingham in order to identify potential drug targets. Previously obtained spatially resolved bulk RNA profiling data from 10 patients was used to identify potential targets suitable for molecular docking analyses (Andrieux *et al.*, 2023). Samples from 5 intra-tumour regions, comprising the core, peripheral rim, invasive margin, 5-ALA positive and 5-ALA negative regions, were obtained from each patient, and RNA sequencing was conducted on these samples (Andrieux *et al.*, 2023). RNA-seq data from the 5-ALA positive population in the invasive margin was compared against data from the tumour core and the 5-ALA negative population using the limma R/Bioconductor statistical

package to identify differentially expressed genes (Ritchie *et al.*, 2015). From this analysis, 141 uniquely expressed genes were upregulated in the 5-ALA positive population relative to the tumour core and 5-ALA negative population.

These genes were run through STRING to visualise protein connectivity and Metascape to identify upregulated biological processes. Kaplan Meier survival analyses for OS and PFS were conducted using cBioportal to identify the clinical relevance of these genes. Six genes were identified, and the binding pockets in the corresponding proteins were identified in their 3D crystal structure. No binding pockets for *KRT79* were identified so this gene was not progressed any further. DrugRep was used to conduct targeted docking of approved and experimental drugs against these binding pockets. Drugs that were predicted to bind to the binding pockets were filtered according to their binding energy score and rotatable bonds for PyRx docking. Drug candidates that passed these filtration steps were then run through PyRx for multiple ligand docking to determine binding affinity and root mean square deviation (RMSD) value for the best confirmation. Drugs that passed this process were further narrowed down according to the following criteria listed in Table 2.1.

Table 2.1: Criteria used to narrow down candidate drugs obtained from PyRx docking for *in vitro* testing.

Post-PyRx Screening	
Binding affinity	≤ -7
Upper and lower RMSD value	0
Rotatable bonds	5 - 15
Previous evidence of cytotoxicity	Yes
Previous use in GBM clinical trials	No
Pre-clinical efficacy in GBM	No
Hydrogen bond donors	≤ 3
Hydrogen bond acceptors	≤ 7
LogP	1.0 – 3.5
Molecular weight (Da)	150 – 450

2.2.2 Proteomic-Based Drug Selection

The following work was carried out by Phoebe McCrorie, Harry Porter, and Shaylen Mistry at the University of Nottingham to identify proteomic-derived drug targets in GIN cell lines. GIN8, GIN28 and GIN31 primary cell lines (derived from the GBM infiltrative margin) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% foetal bovine serum (FBS), 1 g/L D-Glucose and L-Glutamine and pyruvate. Commercial human cortical astrocytes (ScienCell, SC-1800) isolated from the cerebral cortex of a human brain were cultured in Poly-L lysine-coated cell culture flasks (ThermoFisher Scientific). They were cultured in astrocyte media (ScienCell) containing 1x astrocyte growth supplement and 2% FBS to 10 passages. All cell lines were incubated under the same conditions, 37°C with 5% CO₂. Cellular and membrane protein was extracted following the same protocol described below in section 2.6.4. Protein concentrations were quantified using a bicinchoninic acid assay, where bovine serum albumin (BSA) standards ranging from 0 mg/mL to 2 mg/mL were prepared. The cell samples were diluted 1:10, and 10 µL of each standard and sample was added in triplicate to a clear 96-well plate. The bicinchoninic acid reagents were added according to the manufacturer's instructions and then incubated at 37°C for 30 minutes before absorbance was measured at 562 nm using a BMG LabTech Plate Reader with SpectroStar Nano Software. Protein concentrations were calculated against a standard curve using linear regression in Microsoft Excel. 50 µg of total and membrane protein cell lysates for each cell line were outsourced to Nottingham Trent University for LC-MS.

Differentially expressed proteins between the GIN cell lines and human astrocytes were identified using Amica (version 3.0.1) using the limma package with log₂fold-change (log₂FC) >2 thresholds and an adjusted p-value <0.05. This identified 17 commonly upregulated membrane proteins across all three GIN cell lines, which are EDIL3, DYSF, A2M, SERPINE2, RAC2, CD58, PZP, CD70, LOXL1, SLC39A14, PDK, SH3KBP1, APOL2, ASPSCR1, SPTLC3, CDV3 and ROBO1. The 3D crystal structure of each protein was used in DrugRep to conduct targeted docking of approved drugs by Maria Shah. The top 10 most likely proteins to bind were identified for each protein. Maria Shah also conducted the PyRx docking of drugs that had passed the first

filtration step after being identified in DrugRep to identify the best ligand binding conformation.

2.2.3 Metabolomic-Based Drug Selection

The following work was carried out by Rui Chen at the University of Nottingham to identify dysregulated metabolic pathways and potential therapeutics that may target these. Primary cell lines derived from the GBM core enhancing (GCE) region, GCE28 and GCE31, were compared against GIN cell lines derived from the invasive margin, GIN28 and GIN31, using conventional LC-MS-based metabolic profiling (Chen *et al.*, 2026). This identified glycerophospholipid metabolism as being significantly different between the two regions (Chen *et al.*, 2026). This was confirmed by using the single-cell metabolomics techniques, 3D Orbitrap secondary ion mass spectrometry, atmospheric pressure-matrix-assisted laser desorption/ionisation MS, and capillary-flow LC-MS-based metabolite profiling.

A literature review was conducted to identify commercially available inhibitors against each metabolic enzyme within the glycerophospholipid metabolism pathway. Potential therapeutic candidates are shown in Table 2.4. These inhibitors were then filtered based on their previous assessment in GBM research and other cancers. RSM932A was identified as being the best candidate as it conferred reduced tumour growth in patient xenografts for various cancers and was shown to be effective in U87 human GBM cell lines. It is less toxic and has a better safety profile compared to other candidates for the biosynthesis process (Lacal and Campos, 2015). MAFP was selected as it targets both the remodelling and degradation processes through Phospholipase A2 (PLA₂) and Phospholipase B (PLB) (Aaltonen *et al.*, 2020). U-73122 suppresses cell proliferation and promotes apoptosis in IDH wild-type low-grade glioma cells and has been shown to suppress cell invasion in GBM cells but has not been tested in patient-derived cell lines (Khoshyomn *et al.*, 1999; Li *et al.*, 2021).

Table 2.2: Targets within the glycerophospholipid metabolism pathway and commercially available inhibitors. GPAT = Glycerol-3-phosphate acyltransferase. CK = Choline kinase. PLC = Phospholipase C. PLD = Phospholipase D.

Biosynthesis Process	
Targets	Inhibitors
GPAT	FSG67
CK	RSM932A
Remodelling Process	
Targets	Inhibitors
PLA ₂	MAFP
Degradation Process	
Targets	Inhibitors
PLA ₂ and PLB	MAFP
PLC	U-73122, D609
PLD	VU0155069

2.3 Cell Culture

2.3.1 Culturing GIN Cell Lines

GIN8, GIN28 and GIN31 cells were cultured in DMEM supplemented with 1 g/L D-Glucose and L-Glutamine, pyruvate, and 10% FBS at 37°C with 5% CO₂. Cells were cultured in T-25 flasks and passaged when reaching 70-90% confluent. Cells were washed with Hanks' Balanced Salt Solution (HBSS; Gibco) before 3 mL of trypsin-EDTA (ethylenediaminetetraacetic acid; Gibco) was added to detach the cells. The flasks were incubated for 5 minutes at 37°C with 5% CO₂. Cells were seeded into new cell culture flasks at a split ratio of 1:2 – 1:6, depending on cell density and future work.

2.3.2 Cell Counting

Cells were counted using the EVE™ Automatic Cell Counter from NanoEnTek. Following the cell culture method described above, cells were collected from the flasks and centrifuged at 150 x g for 5 minutes in an Eppendorf centrifuge 5702. The supernatant was aspirated, and the cell pellet was resuspended in 3 mL DMEM with 10% FBS. In a new Eppendorf, 10 µL of the cell mixture was stained with 10 µL of

trypan blue and 10 μ L of the trypan blue-stained cell mixture was loaded into an EVE™ cell counting slide, which counted the number of live cells per mL.

$$\text{Amount of cell stock required} = \frac{\text{Number of cells needed in total volume}}{\text{Live cell per mL}}$$

2.4 Drug Selection

2.4.1 Transcriptomic-Derived Drug Selection

The transcriptomic-derived candidate drugs generated through the PyRx screening in Section 2.2.1 were narrowed down based on the criteria in Table 2.1. Idelalisib (Cal-101; S2226), domperidone (S2461) and carvedilol (S1831) were purchased from Selleck Chemicals. Idelalisib and carvedilol were made up to 200 mM in 100% dimethyl sulfoxide (DMSO), and domperidone was made up to 100 mM in 100% DMSO.

2.4.2 Proteomic-Derived Drug Selection

The candidate drugs generated from the DrugRep screening in Section 2.2.2 were narrowed down based on the criteria in Table 2.2.

Table 2.3: Criteria used to select drug candidates for PyRx molecular docking.

Criteria	
Binding energy score (kcal/mol)	≤ -7
Rotatable bonds	5 – 10

Candidate drugs that passed these filtration steps were next screened in PyRx to determine the best binding conformation. The remaining drugs were filtered based on the criteria in Table 2.3.

Table 2.4: Criteria used to filter candidate drugs identified through PyRx docking.

Post-PyRx Screening	
Binding affinity	≤ -7
Previous evidence of cytotoxicity	Yes
Previous use in GBM clinical trials	No
Hydrogen bond donors	≤ 3
Hydrogen bond acceptors	≤ 7
LogP	1.0 – 4.6
Molecular weight (Da)	150 – 550

Lurasidone (HY-B0032A-10mg) and darifenacin (T62320-10 mg) were purchased from Cambridge BioScience and made up to 28 mM and 200 mM, respectively, in 100% DMSO. Nilotinib (SML3799) was purchased from Merck and made up to 100 mM in 100% DMSO.

2.4.3 Metabolomic-Derived Drug Selection

The final candidates were selected through literature reviews carried out in section 2.2.3. U-73122 (HY-13419-10mg), RSM-932A (HY-101144-10mg) and MAFP (HY-10334-5mg) were purchased from MedChem Express. U-73122 and RSM-932A were made up to 125 mM in 100% DMSO. MAFP was made up to 500 mM in 100% DMSO.

2.5 Drug Screening

2.5.1 Metabolic Activity Assay

The Presto Blue™ cell viability assay (ThermoFisher Scientific) was used to measure the metabolic activity of cells to determine cell viability after the addition of various therapeutics. The Presto Blue™ reagent is taken up by cells as blue non-fluorescent resazurin, which is reduced to red, fluorescent resorufin in metabolically active cells. A decrease in fluorescence indicates a decrease in metabolic activity and there cell viability. GIN8, GIN28 and GIN31 cells were seeded at 5000 cells per well in DMEM supplemented with 10% FBS in a transparent 96-well plate. These were incubated overnight at 37°C with 5% CO₂. The existing media was aspirated and replaced with 100 µL of drug-spiked DMEM supplemented with 10% FBS and left for 72 hours at 37°C with 5% CO₂. A blank control was used to determine any background noise, and a vehicle control containing 0.1% DMSO was used. The drug-spiked media was aspirated, and the Presto Blue™ reagent was diluted 1:10 in PBS before 100 µL was added to each well. The plates were incubated in the dark for 45 minutes at 37°C with 5% CO₂. Fluorescence was measured at an excitation of 544 nm and emission of 590 nm using an Infinite 200PRO plate reader with Tecan i-control software. The metabolic activity was determined by deducting the blank control from each condition, and then the data was normalised to the vehicle control.

2.5.2 Luminescent Cell Viability Assay

The Cell Titre Glo assay (Promega) was used to measure cell viability in 3D spheroids, as it displays better penetration than the Presto Blue™ assay. Cell Titre Glo quantifies the amount of ATP released from lysed cells using luminescence as a measure of metabolic activity and cell viability. GIN8, GIN28 and GIN31 cells were seeded at 5000 cells per well in DMEM supplemented with 10% FBS in an ultra-low attachment plate (Corning® Costar®) and centrifuged using a Sigma 3-16L centrifuge at 300 x g for 5 minutes. To allow for spheroid formation, the plate was incubated at 37°C with 5% CO₂ overnight. 100 µL of the existing media was carefully removed and replaced with 100 µL of drug-spiked DMEM supplemented with 10% FBS alongside a vehicle control of 0.1% DMSO. The cells were incubated for 72 hours at 37°C with 5% CO₂. Cell Titre Glo reagent and the plate were equilibrated to room temperature for 30 minutes. 150 µL of the treatment was removed, and 50 µL of the reagent was added. The plate was covered and shaken in a microplate shaker (ThermoScientific) at 500 rpm for 5 minutes. The plate was incubated in the dark at room temperature for 25 minutes before each well was mixed and transferred to a white 96-well plate. The luminescence was read using an Infinite 200 PRO plate reader with Tecan i-control software. To determine cell viability, the data was normalised to the vehicle control.

2.5.3 Synergy Assay

A synergy assay was used to assess the effects of combining RSM932A and U-73122. GIN28 and GIN31 cells were seeded at 5000 cells per well in DMEM supplemented with 10% FBS in a transparent 96-well plate and incubated overnight at 37°C with 5% CO₂. The drugs RSM932A and U-73122 were made up to 4X the desired concentration, and 50 µL of each drug was added to each well. The final concentrations of RSM932A used were 1.25 µM, 2.5 µM, 5 µM and 10 µM. The final concentrations of U-73122 used were 1.25 µM, 2.5 µM, 5 µM, 10 µM and 20 µM. Each concentration of RSM932A was used in combination with each concentration of U-73122. A 0.1% DMSO vehicle control and a blank control were used. The plates were incubated for 72 hours at 37°C with 5% CO₂. The media was aspirated, and the Presto Blue™ cell viability assay was conducted as described above. After the data was normalised, the cell viability data was uploaded to SynergyFinder to assess the effects of combining RSM932A and U-73122 (Zheng *et al.*, 2022).

2.5.4 Transwell Invasion Assay

A transwell invasion assay was used to study whether RSM932A and U-73122 inhibited GBM invasive margin cell invasion, which was adapted from Justus *et al.* (2023) and optimised by Chen *et al.* (2026). Cell media in the cell culture flasks was changed from DMEM supplemented with 10% FBS to FBS-free DMEM 24 hours before the experiment. ThinCert 8 μm cell culture inserts (Greiner) were washed with phosphate-buffered saline (PBS; Gibco) and added to a 24-well plate. Before the experiment, the mouse collagen IV (Cultrex, Trevigen, Bio-Techne Ltd R&D systems) was diluted to 0.5 $\mu\text{g}/\mu\text{L}$ in PBS. During the experiment, the mouse collagen was further diluted to 0.25 $\mu\text{g}/\mu\text{L}$ in PBS, and 60 μL of this solution was used to coat each insert. The plates were left on a rocker overnight at 37°C to allow the collagen matrix to set. The inserts were then soaked with 100 μL FBS-free DMEM for 1 hour, then the drugs RSM932A and U-73122 were made up to 3X the concentration in FBS-free DMEM and 100 μL was added to each insert. GIN28 cells were seeded at 14,000 cells per insert, and GIN31 cells were seeded at 12,000 cells per insert. Each condition was carried out in triplicate; the final concentrations used for each cell line are listed in Table 2.5.

Table 2.5: The final concentrations for RSM932A and U-73122 used in the invasion assay for the GIN28 and GIN31 cell lines.

Condition	Concentration required for GIN28 (μM)	Concentration required for GIN31 (μM)
RSM932A IC ₂₅	1.7	3.9
RSM932A IC ₅₀	3.1	6.4
U-73122 IC ₂₅	3	6.6
U-73122 IC ₅₀	10	14.4
Combination IC ₂₅	1.25 RSM932A + 2.5 U-73122	2.5 RSM932A + 1.25 U-73122
Combination IC ₅₀	1.25 RSM932A + 5 U-73122	1.25 RSM932A + 10 U-73122

At the bottom of each well, 700 μL of DMEM with 10% FBS was added, and the plates were left overnight at 37°C with 5% CO₂. After 24 hours, the media was removed from the insert, and the insert was transferred to new plates filled with 700 μL of methanol to fix the cells. The inserts were left for 1 hour at room temperature, then moved to new plates containing 700 μL of 0.2% crystal violet to stain the cells. Plates were covered with foil and left for 15 minutes. The inserts were washed twice with distilled

water, and the top layer of cells on each insert was removed with a cotton bud before leaving them overnight in a fume hood to dry. The membrane was cut from the insert and placed on a microscope slide. Four different fields of view were imaged for each membrane on a multi-viewing microscope (Brunel N-150), and the number of cells in each image was counted using ImageJ to mark each cell. The average number of cells across the four fields of view was calculated for each membrane and used to calculate the average number of cells that have invaded through the matrix for each condition. The number of cells that had invaded through was normalised to a vehicle control condition of 0.05% DMSO.

2.6 Tumour Tissue Methods

2.6.1 Patient Information and Primary Tissue Sample Collection

This study included 6 patient tumour samples, 5 of which were primary GBMs with no previous treatment and 1 recurrent GBM with prior treatment. All patients were operated on by a single surgeon at Queen's Medical Centre, Nottingham, using the procedure outlined by Smith *et al.* (2020). Samples were collected after informed consent was obtained from human participants. All samples were handled and monitored in accordance with national guidelines of the UK Human Tissue Act 2004, specifically 'Part 2 – Regulation of activities involving human tissue'. 5-ALA was administered orally to patients before the surgery. The samples were taken from the furthest areas of 5-ALA-induced fluorescence where the tumour invaded into the healthy brain parenchyma.

2.6.2 Glioblastoma Tumour Dissociation

The tissue samples were dissociated using a previously optimised protocol (Hussein *et al.*, 2010; Smith *et al.*, 2020; Andrieux *et al.*, 2023). Before tissue dissociation, the enzyme stock solution was prepared at 10x the working concentration in Hanks' Balanced Salt Solution (HBSS; Gibco). The solution contained DNase Type 1 (Worthington) at 1.26 mg/mL, Neutral Protease (Dispase; Worthington) at 0.3 mg/mL, Collagenase Type 1a (Worthington) at 1.28 mg/mL, and Hyaluronidase (Worthington) at 10 mg/mL. This solution was diluted 1:10 with HBSS. The samples were weighed before dissociation, then any blood clots were removed before the sample was minced

using a pre-sterilised single-edge blade. The tissue and diluted enzyme solution were transferred to a T25 cell culture flask and incubated vertically for 30 minutes at 37°C, gently pipetting the solution every 5 minutes. When 80-90% of the tissue had dissolved, the disaggregated cells were collected using a 40 µm cell strainer and rinsed with low glucose DMEM supplemented with 40% FBS, L-glutamine, and penicillin/streptomycin. This was centrifuged at 180 x g for 5 minutes using a Sigma 3-16 centrifuge. The supernatant was discarded, and the pellet was resuspended in 630 µL of 0.5% Bovine Serum Albumin (BSA; Sigma) in 1× PBS buffer and 70 µL of Miltenyi Myelin removal beads (Miltenyi Biotec) per 500 mg of tissue. The sample was incubated on ice for 15 minutes, washed with 7 mL of 0.5% BSA in 1× PBS buffer per 500 mg of tissue, and centrifuged again at 180 x g for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 1 mL of 0.5% BSA in 1× PBS buffer per 500 mg of tissue. LS columns (Miltenyi Biotec) were used to remove myelin, and the remaining cells were collected. Each column can hold up to 500 mg of tissue and was prepared by adding 3 mL of 0.5% BSA in 1× PBS buffer. The cell suspension was added and washed twice with 1 mL of 0.5% BSA in 1× PBS buffer. The collected cells were centrifuged at 180 x g for 5 minutes, and the supernatant was discarded. The pellet was resuspended in 1 mL of fluorescence-activated cell sorting (FACS) staining solution, composed of HBSS supplemented with 1% BSA (w/v), 1 mM EDTA (Sigma), and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Sigma). The samples were stored on ice until FACS.

2.6.3 Fluorescence-Activated Cell Sorting

The invasive margin samples were subjected to FACS, previously optimised by Smith *et al.* (2020). Samples were sorted using the Astrios EQ (Beckman Coulter) with an excitation spectrum at 405 nm and an emission spectrum at 605-625 nm. To set the gates for sorting, U251 GBM cells were used as controls and incubated at 37°C for 2 hours with and without the addition of 5-ALA. U251 GBM cells are a commercially available GBM cell line that fluoresces with the addition of 5-ALA. The cells were sorted into 5-ALA fluorescent positive and 5-ALA fluorescent negative populations before being centrifuged at 500 x g for 5 minutes in a Sigma 1-14 centrifuge. Samples were then snap frozen and stored at -80°C until further use.

2.6.4 Extraction of Membrane and Cytosolic Proteins

Membrane proteins were extracted using a plasma membrane protein extraction kit (ab65400; Abcam). The homogenise buffer was prepared by adding 10 μ L of protease inhibitor to 5 mL of the buffer solution. The samples were resuspended in 1 mL of homogenise buffer, homogenised on ice using a SHM1 handheld homogeniser (Stuart®), and then centrifuged at 700 x g for 10 minutes at 4°C using a Sigma 3-18K centrifuge. The supernatant was collected and centrifuged again at 10,000 x g for 30 minutes at 4°C to separate the cytosolic and membrane proteins. The supernatant containing cytosolic proteins was collected, and the remaining pellet containing membrane proteins was resuspended in 50 μ L of 0.5% Triton X-100 with PBS.

2.6.5 Protein Quantification Using a Bradford Assay

Protein concentration was determined using a Bradford assay (Bradford, 1976). The membrane and cytosolic proteins were mixed with 60 μ L and 100 μ L of homogenise buffer, respectively, and left on ice for 15 minutes. BSA standards were prepared in increasing concentrations from 0 μ g/mL to 100 μ g/mL. The samples were diluted 1:10 with distilled water. Using a 96-well plate, 50 μ L of each standard and sample was added in triplicate. The Bradford reagent (Bio-Rad) was diluted 1:4 with distilled water, and 200 μ L was added to each well. The plate was incubated at room temperature for 5 minutes. The absorbance was measured at 595 nm using a BMG LabTech FLUOstar Omega Plate Reader with Omega software version 5.11 R4. Protein concentrations were determined against the standard curve generated through linear regression in Microsoft Excel.

2.6.6 Liquid Chromatography-Mass Spectrometry Proteomics

LC-MS was outsourced to Nottingham Trent University for the identification and quantification of specific protein targets. 40 μ g of cell lysates from both the total protein and the cytosolic protein samples were used. Due to lower concentrations of membrane proteins extracted from the samples, only 10 μ g of membrane protein was used for each sample.

2.7 Statistical Analysis

Unless otherwise stated, all experiments were carried out in triplicate ($n=3 \pm \text{SEM}$), where n is the number of independent repeats carried out. All data was normalised to the vehicle control. All data analysis was carried out using GraphPad Prism 10 (version 10.5.0). Non-linear regression was performed in GraphPad Prism to generate the inhibition-response curves and IC_{50} values. Standard error of the mean (SEM) was obtained using row statistics. Data was tested for normality using a Shapiro-Wilk normality test with a value of $p>0.05$, indicating normal distribution for all data. To compare data against a control, a one-way ANOVA was used with a Dunnett multiple comparisons post-hoc test. A value of $p<0.05$ was considered statistically significant.

3. Results

Previous unpublished research from our laboratory identified potential drug targets against the GBM invasive margin. During the present study, *in silico* molecular docking identified potential therapeutic candidates that could bind to these targets. These candidates were evaluated and selected based on their properties and the existing literature to increase the chances of identifying an effective compound. Final candidates were then screened using various *in vitro* assays to assess the cytotoxic effects of the drug. GIN cell lines were used in the assays, as these are representative of the residual disease left in the invasive margin and adjacent brain parenchyma, and associated with recurrence.

3.1 Transcriptomic-Based Drug Screening

Previous unpublished work within our group identified five clinically relevant genes within the 5-ALA positive invasive margin. Kaplan Meier survival curves were created using cBioportal to determine the association of these genes with both OS and PFS. These included *SUN3* for OS, *RENBP* and *MRGPRX3* for both OS and PFS and *ZNF185* and *FOSL1* for PFS.

3.1.1 Selection of Transcriptomic Drug Candidates

PyRx docking produced 577 approved drugs that could bind to the protein produced by these five clinically relevant genes. Using the selection criteria described in Table 2.1 to narrow these drugs down, three final drug candidates were identified: idelalisib, domperidone and carvedilol (Table 3.1). Idelalisib potentially binds to binding pocket 1 of the protein *RENBP*. Carvedilol potentially binds to binding pocket 1 of the protein *ZNF185*. Domperidone could potentially bind to pocket 1 of *ZNF185*, pocket 2 of *MRGPRX3*, and pocket 4 of *RENBP* proteins.

Table 3.1: Drug candidates that were identified through PyRx docking and fit the criteria for *in vitro* drug testing. RMSD = root mean square deviation. ¹ = ZNF185 binding pocket 1, ² = MRGPRX3 binding pocket 2, ³ = RENBP binding pocket 4.

Criteria	Idelalisib	Domperidone	Carvedilol
Upper and lower RMSD value	0	0	0
Binding affinity	-9.6	-8.6 ¹ , -8.8 ² , -7.9 ³	-9.4
Rotatable bonds	5	5	6
Previous evidence of cytotoxicity	Yes	Yes	Yes
Previous use in clinical trials	No	No	No
Pre-clinical efficacy in GBM	No	No	No
Hydrogen bond donors	2	2	3
Hydrogen bond acceptors	6	3	5
LogP	3.36	2.9	3.42
Molecular weight (Da)	415.43	425.91	406.47

3.1.2 Cytotoxicity of Idelalisib, Domperidone and Carvedilol Against GBM Invasive Margin Cells

This study aimed to assess the cytotoxicity of potential candidate therapeutics against the GBM invasive margin. To assess if these therapeutics confer a cytotoxic effect, Presto Blue™ assays were used. A Presto Blue™ assay uses fluorescence to measure the metabolic activity of cells; a decrease in the fluorescence indicates a decrease in metabolic activity and therefore cell viability. A panel of three GIN cell lines derived from the invasive tumour margin was used in this assay to evaluate the three lead drug candidates. The GIN cell lines used were GIN8, GIN28 and GIN31. The results of this drug screening are shown in Figure 3.1.

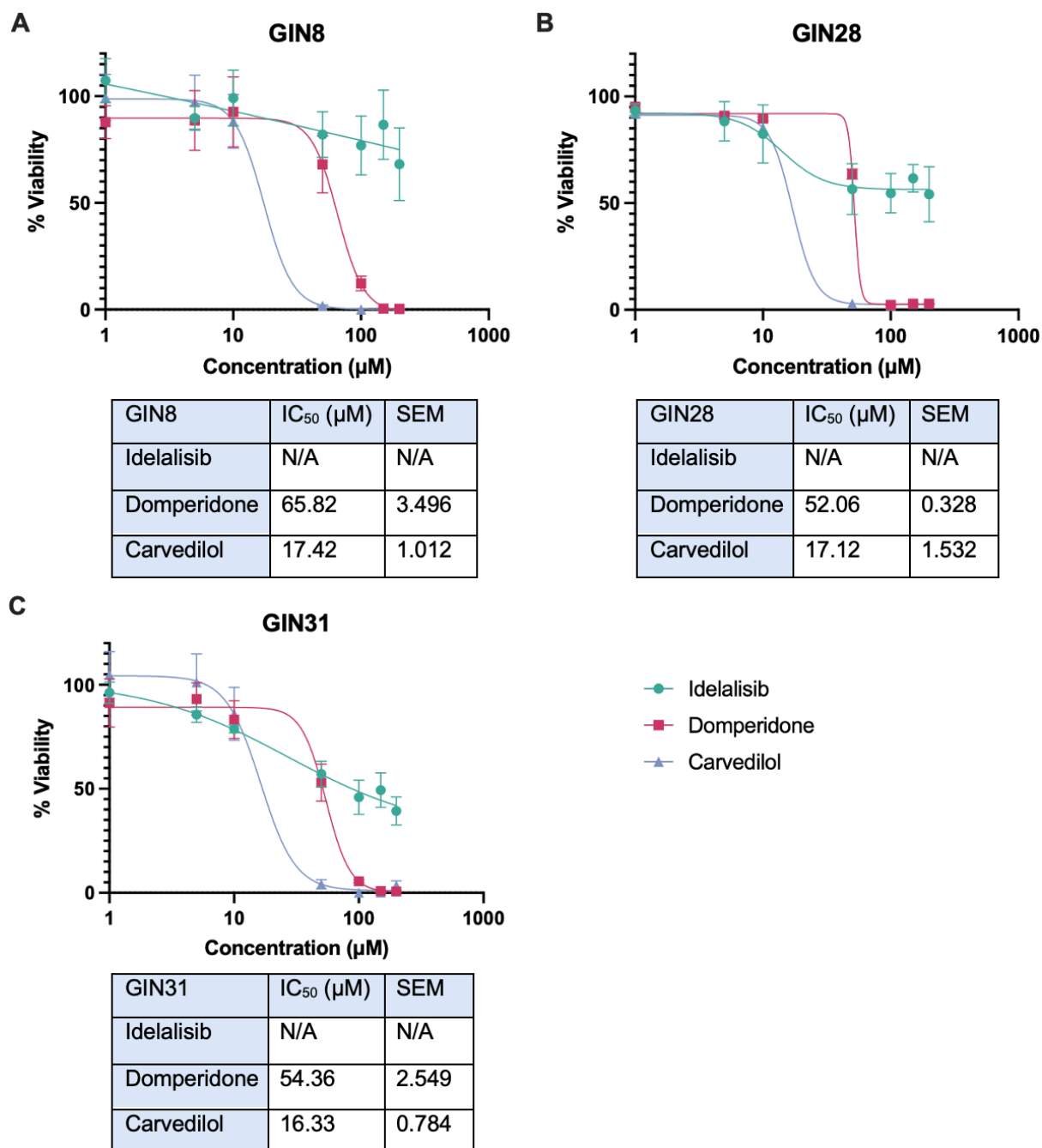


Figure 3.1: Dose response curves and IC₅₀ values for GIN8 (A), GIN28 (B) and GIN31 (C) cell lines incubated for 72 hours with idelalisib, domperidone and carvedilol. Cells were seeded in a 2D monolayer, and each drug was used in increasing concentrations from 1 µM to 200 µM. Cell viability was assessed using a Presto Blue assay. Data was normalised to the carrier control and analysed using the non-linear regression function. N/A signifies the IC₅₀ value was not reached. Data is shown as mean ± SEM, *n*=3. IC₅₀ = Concentration required to inhibit cell viability by 50%.

Figure 3.1 shows that both domperidone and carvedilol produced a dose-dependent decrease in cell viability. Carvedilol was the most potent drug against all three cell lines, with a similar IC₅₀ value in each cell line. Domperidone was potent against all three cell lines, but not as potent as carvedilol. While idelalisib reduced cell viability

somewhat, it did not cause complete cell death at 200 μ M, so an IC₅₀ could not be obtained. Due to this, idelalisib was not taken forward into assay utilising 3D models.

Domperidone and carvedilol were then tested in 3D spheroids as these better mimic the *in vivo* environment while still allowing high-throughput screening to take place. They can exhibit some spatial heterogeneity due to oxygen and nutrient gradients, such as proliferating cells at the rim compared to hypoxic and quiescent cells at the core of the spheroid, which better mimics the *in vivo* tumour (Nath and Devi, 2016; Fang and Eglen, 2017). To determine if domperidone and carvedilol retained potency in 3D spheroids for each GIN cell line, they were tested at the same concentrations as the 2D assay. While Presto Blue can be used to measure cell viability in 2D, it exhibits limited penetration in 3D spheroids, meaning it may not penetrate to the core of the spheroid. The Cell Titre Glo assay lyses cells, and the luminescence measured indicates the amount of ATP present from metabolically active cells; a decrease in luminescence indicates a reduction in cell viability. Therefore, Cell Titre Glo was used to measure cell viability in 3D spheroids because it exhibits enhanced penetration. The results are shown in Figure 3.2.

Both domperidone and carvedilol caused dose-dependent decreases in cell viability in all three GIN cell lines when seeded as 3D spheroids. Domperidone was the most potent drug when tested in 3D models of the GIN28 cell line, contrary to what was observed in the 2D monolayer assay (Figure 3.1). Carvedilol remained the most potent drug in GIN8 and GIN31 cell lines. While it remained the most potent, the IC₅₀ values for carvedilol in these cell lines increased over 2-fold in the 3D models compared to the IC₅₀ values produced when tested in 2D monolayers. The IC₅₀ values produced when testing domperidone in 3D models of GIN8 and GIN31 cell lines remained similar to the values produced when tested in 2D monolayers of these same cell lines. When domperidone was tested in 3D models of GIN28, the IC₅₀ value decreased nearly 3-fold.

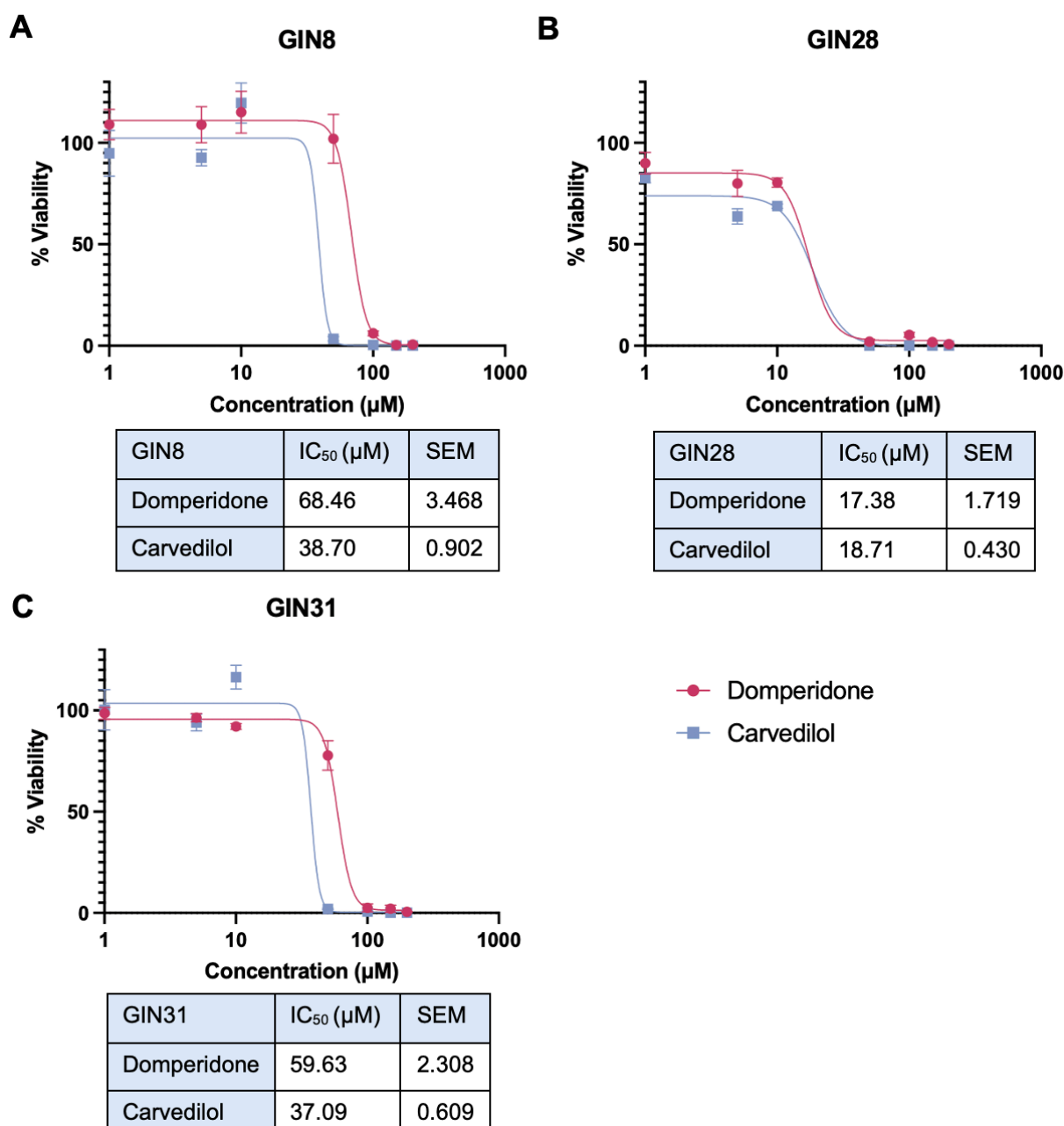


Figure 3.2: Dose response curves and IC₅₀ values for 3D spheroids of GIN8 (A), GIN28 (B) and GIN31 (C) cell lines incubated with domperidone and carvedilol for 72 hours. 3D spheroids for each cell line were generated and incubated with increasing concentrations of each drug from 1 µM to 200 µM. Cell viability was assessed using a Cell Titre Glo assay. Data was normalised to the carrier control and analysed using the non-linear regression function. Data is shown as mean ± SEM, *n*=3.

3.2 Proteomic-Based Drug Screening

Previous unpublished research in the group identified 17 commonly upregulated membrane proteins in GIN cell lines when compared to healthy human astrocytes. These proteins were EDIL3, DYSF, A2M, SERPINE2, RAC2, CD58, PZP, CD70, LOXL1, SLC39A14, PDK, SH3KBP1, APOL2, ASPSCR1, SPTLC3, CDV3 and ROBO1.

3.2.1 Selection of Proteomic-Based Drug Candidates

DrugRep identified the top 10 drugs predicted to bind to each protein, based on binding energy. The top 10 drugs were filtered based on their binding energy and the number of rotatable bonds within the chemical structure. The binding energy indicates how likely a drug is to bind with its intended target. A lower binding energy value indicates a favourable binding affinity, increasing the likelihood that a drug will bind and interact with its target. A value below -7 kcal/mol is regarded as having high-affinity binding, increasing the chances of finding a successful candidate. The number of rotatable bonds within a structure dictates how flexible the molecule will be. If a molecule has too many rotatable bonds, it may struggle to maintain the conformation it needs to fit within the binding site. However, if there are not enough rotatable bonds, the molecule may be too rigid. A range of 5 – 10 rotatable bonds was selected as the 'extended rule of five' states that 10 or fewer rotatable bonds would lead to good absorption and permeability of the drug (Veber *et al.*, 2002). The drug candidates, based upon the top 10 drugs for each protein, that underwent PyRx docking are shown in Table 3.2.

Table 3.2: Drug candidates that passed the first filtration step that underwent PyRx docking. N/A signifies no drugs passed the filtration step.

Proteins	Drugs remaining after first filtration
EDIL3	Pimozide, ubrogepant, dihydroergocristine, mizolastine
DYSF	Digoxin, digitoxin, acetyldigitoxin
A2M	Leucovorin, levoleucovorin, avatrombopag, folic acid, elexacaftor, digoxin, lurasidone, sulfasalazine
SERPINE2	Acetyldigitoxin, chlorhexidine, ergocalciferol, ibrutinib, digitoxin
RAC2	Lumateperone
CD58	N/A
PZP	Acetyldigitoxin, digitoxin, cabozantinib, imatinib, naldemedine,
CD70	Tucatinib, delamanid, eltrombopag, troglitazone, levoleucovorin, digoxin
LOXL1	Glecaprevir, entrectinib, nilotinib, dihydroergocristine
SLC39A14	Regorafenib, tucatinib, novobiocin, acetyldigitoxin, delamanid, pimozide, sorafenib
PXK	Ponatinib, acetyldigitoxin, digoxin, tedizolid phosphate, dasatinib
SH3KBP1	Regorafenib, tucatinib, eltrombopag, pimozide, zafirlukast, sorafenib, darifenacin, lumacaftor
APOL2	Acetyldigitoxin, digoxin, ponatinib, aripiprazole, mirabegron, pexidartinib
ASPSCR1	Dexamethasone metasulfobenzoate, regorafenib, sorafenib, leucovorin, pexidartinib, alatrofloxacin, lymecycline
SPTLC3	Raltegravir, lumacaftor, cromoglicic acid, glimepiride, irinotecan, fluspirilene
CDV3	Netarsudil, levoleucovorin, masoprocol, pentamidine arbutamine, troglitazone, crizotinib
ROBO1	Dihydroergocristine, pexidartinib, dihydro-alpha-ergocryptine

After PyRx docking, the drug candidates were filtered based on the binding affinity score. This filtered 86 candidates down to 55. For these 55 candidates, literature searches were conducted to gather evidence of any cytotoxic effects. There was no existing literature on the cytotoxic effect of 10 of these candidates. Out of the remaining 45 candidates, 10 have been tested in clinical trials for GBM, so these were not taken forward as they are not novel candidates. DrugBank was then used to obtain the values for hydrogen bond donors and acceptors, logP and the molecular weight of the

compounds. Lipinski's rule of five states that drugs should have 5 or fewer hydrogen bond donors and 10 or fewer hydrogen bond acceptors (Lipinski *et al.*, 2001). Too many hydrogen bond donors or acceptors may impair a compound's solubility and permeability. A smaller number of hydrogen bond donors, such as 3, and acceptors, such as 7, will increase the chances of the compound displaying good absorption and permeability, increasing the chances of crossing the BBB. The logP value indicates the lipophilicity of a compound and how soluble it will be. A low logP value means the compound may be taken up by the body more easily due to the high water content, but if this value is too low, it may struggle to cross membranes. Lipinski's rule of five states that a logP value greater than 5 can indicate poor absorption or permeability (Lipinski *et al.*, 2001). After filtering the candidate drugs based on these properties, 4 remained; these are shown in Table 3.3

Table 3.3: Potential candidate drugs remaining after filtration following PyRx docking.

Criteria	Lurasidone	Darifenacin	Nilotinib	Dihydroergocristine
Binding energy (kcal/mol)	-7.7	-8	-9.9	-9.7
Rotatable bonds	5	7	7	5
Binding affinity	-8.5	-8.5	-10.6	-10.1
Previous evidence of cytotoxicity	Yes	Yes	Yes	Yes
Use in GBM clinical trials	No	No	No	No
Hydrogen bond donors	0	1	2	3
Hydrogen bond acceptors	5	3	6	6
LogP	4.56	4.54	4.51	3.83
Molecular weight (Da)	492.68	426.55	529.52	611.74

As a final criterion, the compounds were filtered against the molecular weight of the drug to identify 3 lead drug compounds to progress to *in vitro* testing. According to Lipinski's rule of five, a molecular weight of over 500 Da can increase the likelihood of

poor absorption or permeability (Lipinski *et al.*, 2001). As a value of 500 Da would have identified only 2 drug candidates, the criterion for this was increased to 550 Da to produce 3 lead compounds. These three candidates are nilotinib, darifenacin and lurasidone.

3.2.2 Cytotoxicity of Nilotinib, Darifenacin and Lurasidone Against GBM Invasive Margin Cells

The cytotoxic effects of nilotinib, darifenacin and lurasidone were assessed using a Presto Blue™ cell viability assay. A decrease in fluorescence indicates a reduction in metabolic activity and cell viability. These three therapeutics were tested against a panel of three GIN cell lines, GIN8, GIN28 and GIN31, which were seeded as a 2D monolayer. The results of this drug screening are shown in Figure 3.3.

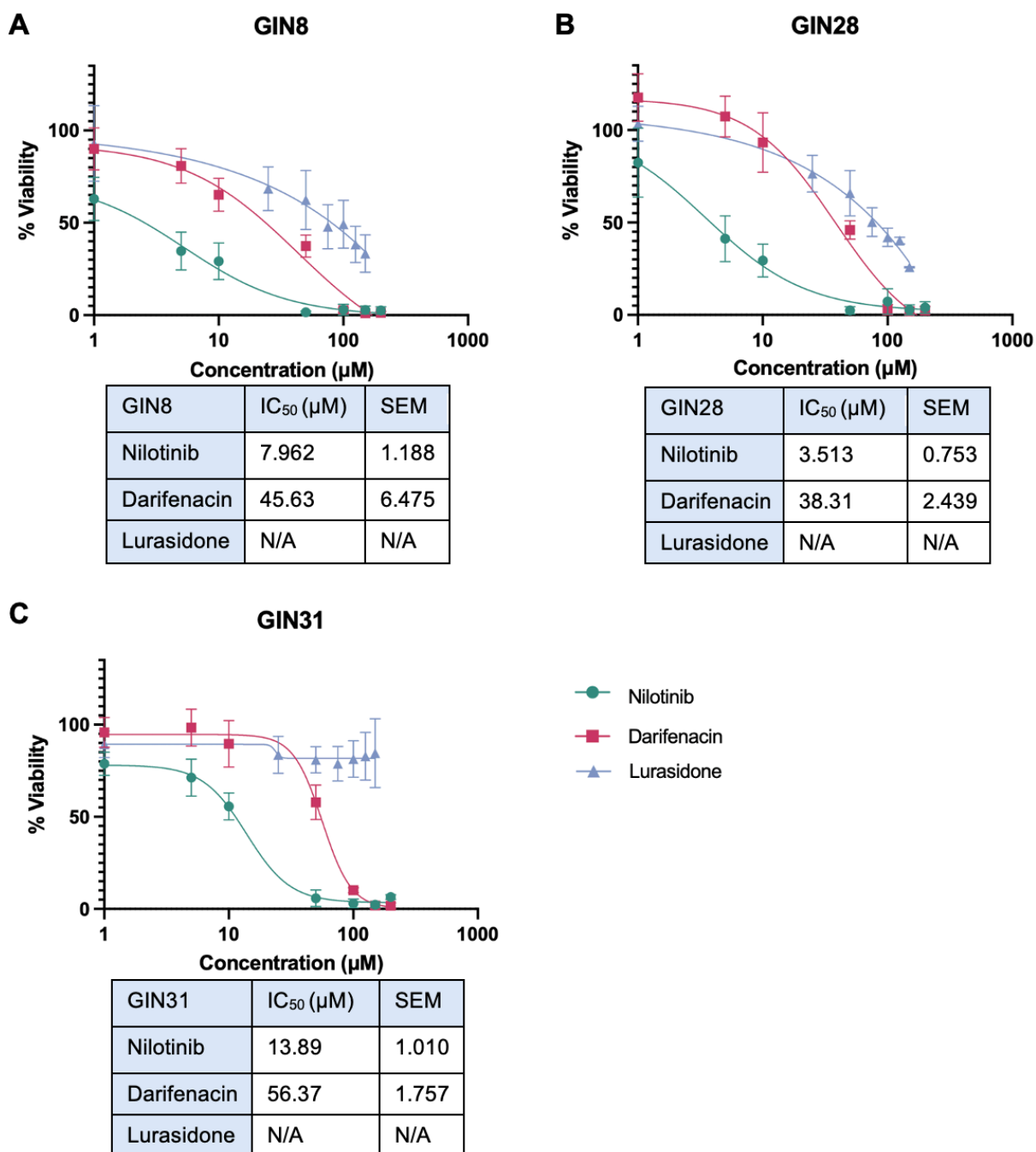


Figure 3.3: Dose response curves and the parameters of nilotinib, darifenacin and lurasidone incubated for 72 hours with GIN8 (A), GIN28 (B) and GIN31 (C) cell lines. Nilotinib and darifenacin were added at increasing concentrations from 1 µM to 200 µM. Lurasidone was added in increasing concentrations from 1 µM to 150 µM. Cell viability was assessed using a Presto Blue assay. Data was normalised to the carrier control and analysed using the non-linear regression function. N/A indicates the IC₅₀ was not reached. Data is shown as mean ± SEM, $n=3$, except GIN8 nilotinib, which is $n=2$.

Nilotinib and darifenacin both produced a dose-dependent decrease in cell viability in all three cell lines. Lurasidone did not reach the IC₅₀ by the 150 µM concentration, so it would not be a good candidate for further studies. The most potent drug against all three cell lines was nilotinib, with the IC₅₀ varying slightly between cell lines (most

potent in GIN28 cells, but the least potent in GIN31 cells). This pattern for IC₅₀ concentrations was also observed with darifenacin, whereby darifenacin was less potent than nilotinib overall, but still produced a response.

3.3 Metabolomic-Based Drug Screening

The glycerophospholipid pathway was previously found to be significantly dysregulated in GIN cells compared to the corresponding GBM tumour core, GCE cells (Chen *et al.*, 2026). Literature reviews against each metabolite within this pathway yielded three potential drug candidates, RSM932A, U-73122, and MAFP. RSM932A targets CK, U-73122 targets PLC, and MAFP targets both PLA₂ and PLB within this pathway. These drugs were tested against 2D monolayers of GIN28 and GIN31 cell lines using a Presto Blue™ assay to determine if there is a reduction in the metabolic activity. These compounds were only tested on these cell lines because the metabolomics work was predicated on the GIN28 and GIN31 cell lines. The results of this drug screen are shown in Figure 3.4.

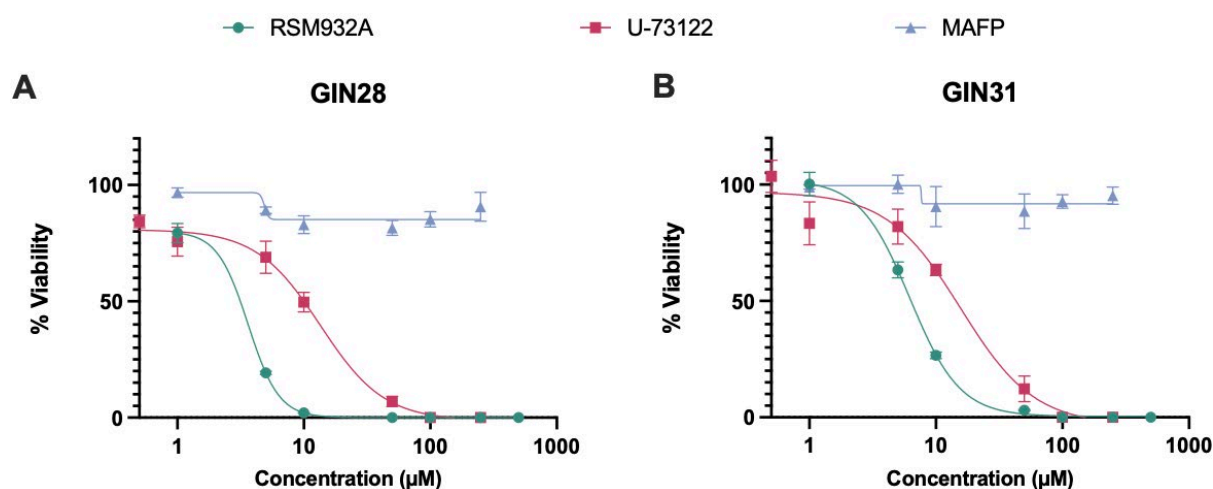


Figure 3.4: Dose response curves for GIN28 (A) and GIN31 (B) cell lines incubated for 72 hours with RSM932A, U-73122 and MAFP. Cells were incubated with increasing concentrations of each drug. RSM932A was used from 1 µM to 500 µM, U-73122 was used from 0.5 µM to 250 µM, and MAFP was used from 1 µM to 250 µM. Cell viability was assessed using a Presto Blue assay. Data was analysed using the non-linear regression function. Data is shown as mean ± SEM, n=3.

Both RSM932A and U-73122 dose-dependently decreased cell viability. RSM932A was the most potent drug against both cell lines. The IC₅₀ values for RSM932A incubated with GIN28 and GIN31 cell lines were 3.624 µM and 6.217 µM, respectively. For U-73122, the IC₅₀ values were 13.45 µM and 15.73 µM for GIN28 and GIN31 cell

lines, respectively. MAFP decreased cell viability very little and did not reach IC₅₀ at the concentrations tested and therefore was not taken any further.

3.3.1 Synergistic Effects of RSM932A and U-73122

As RSM932A and U-73122 proved to be cytotoxic against GIN28 and GIN31 cell lines at very low micromolar concentrations, they were tested for synergistic interactions. The average IC₅₀ value for each drug across both cell lines was calculated, 5 µM for RSM932A and 10 µM for U-73122. Various concentrations around these average values were used in combination against a panel of GIN28 and GIN31 cell lines. Cell viability was measured using a Presto Blue™ assay. The fluorescence was measured and normalised to the carrier control, and this data was then analysed using the web application Synergy Finder Plus to determine the effects of combined RSM932A and U-73122 (Zheng *et al.*, 2022). The results are shown in Figure 3.5.

RSM932A and U-73122 showed no synergistic effect in GIN28 cell lines with an average synergy score of -1.3. Combining these drugs in GIN28 cell lines produces an overall additive effect. The highest additive effect was produced when combining 1.25 µM RSM932A and 20 µM U-73122. However, an antagonist effect was produced when using 1.25 µM RSM932A and 1.25 µM U-73122.

When RSM932A and U-73122 were combined in GIN31 cells, an additive effect was observed, with some values nearing the threshold for synergy. The two highest scores were 8.32 for 2.5 µM RSM932A and 1.25 µM U-73122, and 8.08 for 2.5 µM RSM932A and 10 µM U-73122. In the GIN31 cell lines, there was no antagonistic effect, with the lowest score being -8.98 for 1.25 µM RSM932A and 2.5 µM U-73122.

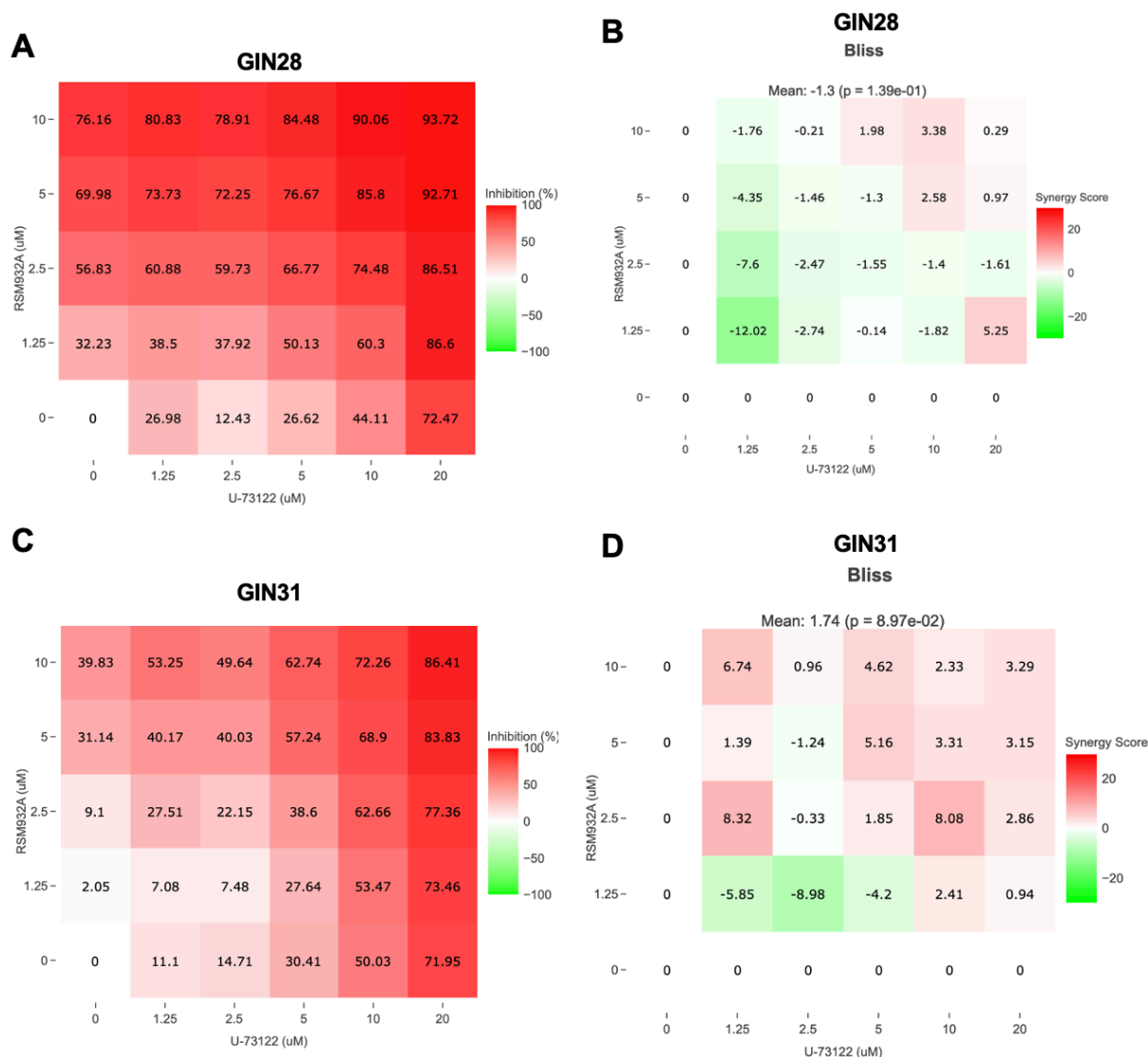


Figure 3.5: The effects of combining RSM932A and U-73122 in GIN28 cells (A-B) and GIN31 cells (C-D). Cell viability was assessed using a Presto Blue assay. (A, C) Represent heatmaps showing growth inhibition of GIN28 cells (A) and GIN31 cells (C) when incubated with varying concentrations of RSM932A and U-73122 for 72 hours. High inhibition is represented as red, with low levels of inhibition as white. (B, D) Bliss synergy scores for RSM932A and U-73122 against GIN28 (B) and GIN31 (D) cell lines. Potential synergistic effects (>10) are shown in red, potential antagonist effects (< -10) are shown in green and potential additive effects ($-10 < x < 10$) are shown in white. Data is the mean score ($n=3$).

3.3.2 Effects of RSM932A and U-73122 on GBM Cell Invasion

RSM932A and U-73122 target the glycerophospholipid pathway, which has been linked to GBM invasion (Koundouros and Poulogiannis, 2019; Korbecki *et al.*, 2023; Chen *et al.*, 2026). Therefore, these drugs were next tested in a transwell invasion assay to determine if they may have anti-invasion properties. Each drug was tested at the IC₂₅ and IC₅₀ concentrations determined from the 2D monolayer cell viability assay (Figure 3.4). They were also tested at the combination IC₂₅ and IC₅₀ concentrations determined from the synergy assay (Figure 3.5). GIN28 and GIN31 cells were seeded

onto collagen-coated inserts and incubated for 24 hours with the various concentrations of RSM932A and U-73122. To determine the number of cells that had invaded through the collagen matrix, four images from each insert were taken, and the number of cells in each image was counted. For each condition, there were three inserts, and the average number of cells that had invaded was calculated and normalised to the vehicle control condition. The results of the invasion assay are shown in Figure 3.6.

The results showed that RSM932A and U-73122 had a small effect on invasion in the GIN28 cell line. Figure 3.6C-D shows that the largest reduction in invasion was observed when U-73122 and both drugs in combination were used at the IC_{50} concentrations. This figure also shows that a larger reduction in invasion was observed in the GIN31 cell line compared to the GIN28 cell line. U-73122 at the IC_{50} concentration and both drugs in combination at the IC_{50} concentration reduced invasion by over 50% in the GIN31 cells. A Shapiro-Wilk test was used to test for normality, with $p > 0.05$. As the data was normally distributed, a one-way ANOVA was used to determine if there was any significant reduction in invasion. A Dunnett multiple comparisons post-hoc test was used to compare each condition against the vehicle control. While a noticeable reduction in invasion in the GIN31 cell line in some conditions can be observed in Figure 3.6D, there was no statistically significant difference in any cell line.

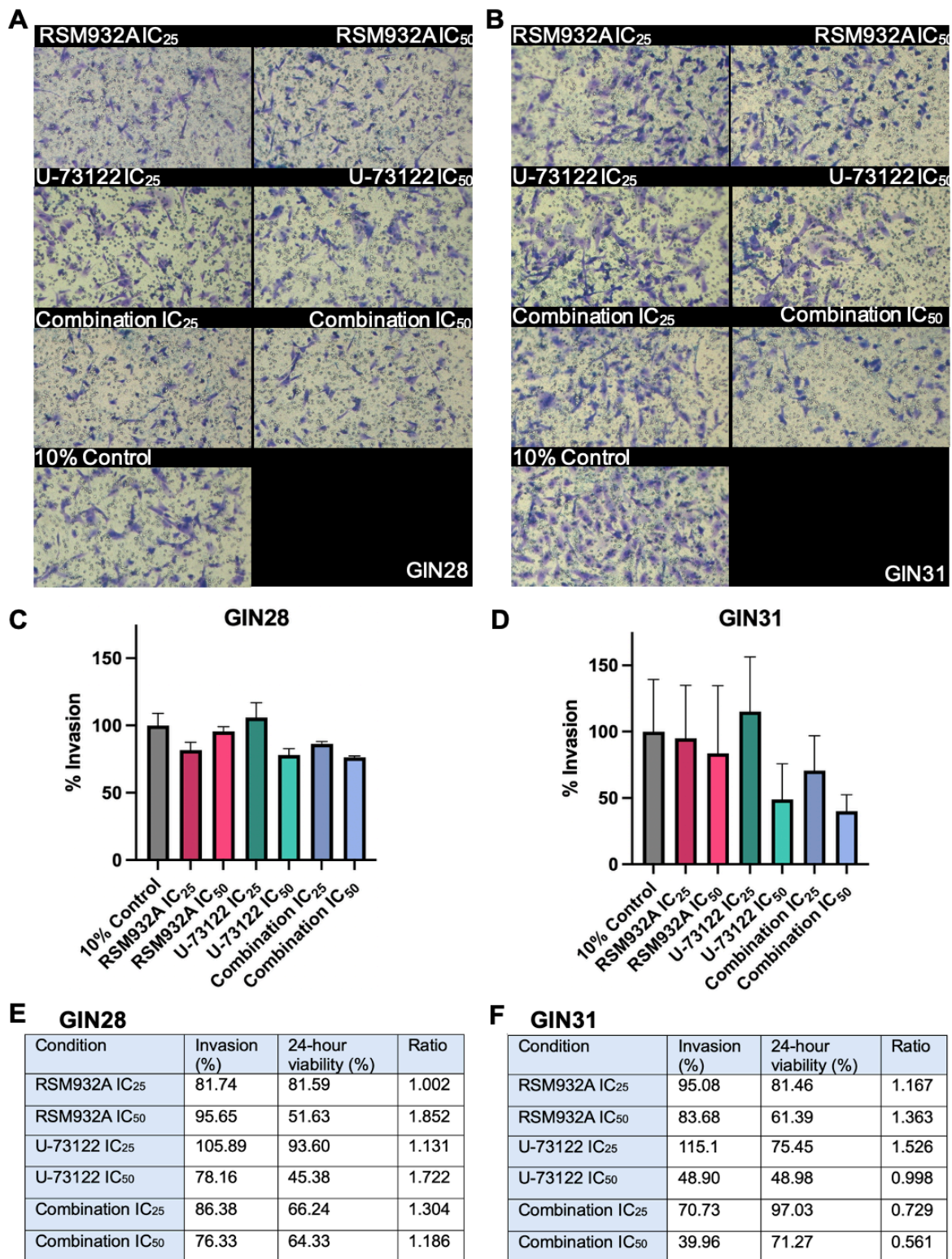


Figure 3.6: The effects of RSM932A and U-73122 on GIN28 and GIN31 cell invasion. GIN28 and GIN31 cells were seeded onto a collagen membrane and incubated with drug-spiked media for 24 hours. (A, B) Images taken of a section of membrane for each condition for GIN28 (A) and GIN31 (B) cells. (C, D) The effects of each condition on invasion in GIN28 (C) and GIN31 (D) cells, normalised to a vehicle control towards 10% FBS. Data is normally distributed according to the Shapiro-Wilk test with $p > 0.05$. No significant differences were found when running a one-way ANOVA with a Dunnett's post-hoc test, where $p < 0.05$. (E, F) Tables displaying invasion and cell viability after a 24-hour incubation with the drug for GIN28 (E) and GIN31 (F) cells. The ratio represents invasion divided by viability to take into account the effect of cell death on invasion. Data is shown as mean $\pm$ SEM, $n=3$.

RSM932A and U-73122 were cytotoxic after a 72-hour incubation with GIN28 and GIN31 cell lines (Figure 3.4). Therefore, a 24-hour viability assay was conducted to assess the effects of both drugs on cell viability in the invasion assay (Figure 3.6E-F). A decrease in cell viability could affect the outcomes observed in the invasion assay. There would be a different number of cells present in each condition which may enable the cells to invade through the matrix easier as there is less competition for space and nutrients. Each cell line was treated with the same concentrations for the same time period used in the invasion assay, and cell viability was measured using a Presto Blue assay. As the Presto Blue assay measures the metabolic activity of cells, a decrease in fluorescence indicates a reduction in the number of live cells. A decrease in cell viability was observed for every concentration in both cell lines. Figure 3.6E shows that the smallest decrease in cell viability was observed at the IC₂₅ concentration of U-73122, and the largest decrease was observed at the IC₅₀ concentration of U-73122 in the GIN28 cell line. Figure 3.6F shows the GIN31 cell line, with the smallest decrease in cell viability observed at the drug combination IC₅₀ and the largest decrease was observed in the U-73122 IC₅₀ condition. Figure 3.6E-F shows a ratio created by dividing the % invasion by the % cell viability to normalise for cell death. When accounting for the reduction in cell viability, a reduction in invasion was not observed for any of the conditions within the GIN28 cell line. Within the GIN31 cell line, a reduction in invasion was observed at the drug combination IC₂₅ and IC₅₀ concentrations and the IC₅₀ concentration for U-73122. The largest reduction in invasion was observed at the drug combination IC₅₀ in GIN31 cells, with a nearly 50% reduction observed.

3.4 Identification of Potential Drug Targets within GBM Invasive Margin Tissue Samples

Identification of proteins present in 5-ALA positive tissue samples from the invasive margin has yet to be achieved and offers a means to clinically validate candidate protein targets of interest identified by *in vitro* studies. This project aims to identify potential drug targets in the form of ubiquitous, differentially expressed proteins in GBM invasive margin tissue samples. The invasive margin tissue samples from 6 patients were collected from surgery and dissociated. Samples from patients 1, 3, 4,

5 and 6 were obtained from primary GBM tumours, while the sample from patient 2 was obtained from a recurrent GBM tumour.

3.4.1 Separation of 5-ALA Positive and Negative Cell Populations

Once the tumour tissue samples were dissociated, five samples were sent to FACS to sort the 5-ALA fluorescent positive cells from non-fluorescent cells. The tissue sample from patient 5 did not undergo FACS and was instead snap frozen after the tissue was dissociated, and the cells were collected. The number of cells collected after FACS for each sample is listed in Table 3.4.

Table 3.4: Number of cells collected from each patient after 5-ALA fluorescent-based FACS.

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 6
5-ALA + cells	750,217	684,634	179,864	150,000	655,976
5-ALA - cells	1,080,713	1,600,000	2,001,746	1,600,000	1,600,000

The 5-ALA positive and negative cells were successfully sorted from the mixed samples. However, because the FACS process took place later in the day than usual, fewer 5-ALA positive cells were collected for patient 3 and patient 4.

3.4.2 Identification of the Contaminant Present in the Samples

Proteins from the sorted samples were extracted and outsourced for LC-MS. Upon running the samples through LC-MS, unusual peaks were observed within the chromatogram that indicated the presence of a contaminant. This was identified as polyethylene glycerol (PEG). This masked the protein peaks and meant individual proteins within the sample could not be quantified. The contaminant was identified using two methods: a manual search and a skyline contamination search. To conduct the manual search, the data obtained from LC-MS was opened in PeakView software, and the unusual peaks were isolated and expanded. These peaks were exported to Excel and manually cross-referenced against a database of known contaminants adapted from Keller *et al.* (2008). The second method followed a study by Gomez-Zepeda *et al.* (2024), which used Skyline to match common background ions with the unusual peaks generated from the samples.

To identify where this contaminant was introduced in our process, a protein sample was precipitated and outsourced, alongside blanks containing the FACS sheath fluid and the extraction buffer used during the protein extraction. When these were run through LC-MS, the contaminant was still present. This led to the idea that the contamination was due to autoclaved Eppendorf tubes or pipette tips that were used during the protein extraction. During sample collection, the patient 5 sample was snap frozen after the tissue dissociation before undergoing FACS. The total protein from this mixed sample, and leftover 5-ALA negative cells from patients 3 and 4, were extracted in a sterile cell culture hood, using new sterile pipette tips and sterile protein lo-bind Eppendorf tubes which had not been autoclaved. The results from these samples showed no contamination and provided some proteomic data.

3.4.3 Identification of Potential Protein Targets Within Tumour Tissue Samples

After identifying where the contaminant came from, these further samples were subjected to LC-MS to determine if they were contaminant-free and could provide some proteomic data. As the 5-ALA negative samples were left over, protein concentration was different between each sample outsourced for LC-MS. From this, the top 10 most abundant proteins present in the mixed patient 5 sample (that were absent from the two 5-ALA negative samples) were identified (Table 3.5). Rows containing a group of multiple proteins signify a peptide was recognised that could belong to any of the proteins within the group.

Table 3.5: The top 10 most abundant proteins present in the mixed patient 5 sample that are absent in the 5-ALA negative samples. POTE = Prostate, ovary, testes expressed.

Gene	Protein Name	Signal Intensity
DST	Dystonin	5938.42
HK1; HK2; HK3; HKDC1	Hexokinase 1; Hexokinase 2; Hexokinase 3; Hexokinase domain containing 1	2026.74
SFN; YWHAB; YWHAE; YWHAH; YWHAQ; YWHAZ	Stratifin; 14-3-3 protein beta; 14-3-3 epsilon; 14-3-3 eta; 14-3-3 protein theta; 14-3-3 protein zeta	1456.67
IGHG1; IGHG2; IGHG3; IGHG4	Immunoglobulin heavy constant gamma 1; Immunoglobulin heavy constant gamma 2; Immunoglobulin heavy constant gamma 3; Immunoglobulin heavy constant gamma 4	1238.53
ACTA1; ACTA2; ACTB; ACTC1; ACTG1; ACTG2; POTEE; POTEF; POTEL; POTEJ	Actin alpha skeletal muscle; Actin aortic smooth muscle; Actin cytoplasmic 1; Actin alpha cardiac muscle 1; Actin cytoplasmic 2; Actin gamma-enteric smooth muscle; POTE ankyrin domain family member E; F; I; J	1008.63
PGK1; PGK2	Phosphoglycerate kinase 1; Phosphoglycerate kinase 2	941.602
HSD17B12	Oestradiol 17-beta-dehydrogenase 12	939.948
MNDA	Myeloid cell nuclear differentiation antigen	895.952
F13A1	Coagulation factor XIII A chain	893.333
CORO2B	Coronin 2B	834.239

The 17 proteins identified from the GIN cell lines were compared against the list of proteins generated from the mixed patient sample and the two 5-ALA negative samples. Proteins identified from the cell lines that were present in any of the three uncontaminated samples are listed in Table 3.6.

Table 3.6: Proteins previously identified in GIN cell lines and their abundance in tumour tissue samples. TP=Total protein. N/A indicates the protein was not detected in that sample.

Gene	Protein name	Signal Intensity		
		Mixed patient 5 TP	Patient 3 5-ALA negative TP	Patient 4 5-ALA negative TP
A2M	Alpha-2-macroglobulin	122.818	739.927	2208.92
APOL2	Apolipoprotein L2	9.25382	N/A	N/A
CD58	Lymphocyte function-associated antigen 3	86.871	N/A	N/A
EDIL3	EGF-like repeats and discoidin domains 3	74.9472	N/A	N/A
PZP	Pregnancy zone protein	N/A	254.537	767.472
RAC1; RAC2; RAC3	Ras-related C3 botulinum toxin substrate 1; Ras-related C3 botulinum toxin substrate 2; Ras-related C3 botulinum toxin substrate 3	1397.1	1863.61	1513.32

Out of the 17 proteins identified from GIN cell lines, only 6 were identified in tumour tissue samples. APOL2, CD58 and EDIL3 were present only in the mixed sample and were absent from both 5-ALA negative samples. Both A2M and the RAC family of proteins, including RAC2, were observed in all three samples. However, PZP was the only protein absent from the mixed sample but present in the 5-ALA negative samples.

4. Discussion

GBM is an aggressive and fatal brain tumour that remains incurable, despite extensive research over several decades. The median survival for GBM is around 15 months despite multimodal standard of care treatment (Thakkar *et al.*, 2014). This tumour is extremely invasive, whereby malignant cells can invade healthy brain tissue. This region is known as the 'invasive margin'. After the primary surgery, these cells remain within the brain, often becoming resistant to radiotherapy and chemotherapeutic agents. These invasive cells are associated with tumour recurrence, which is fatal and typically untreatable. This highlights the need to rapidly identify therapeutics that specifically target this region to prevent recurrence and improve the overall survival of this condition. Repurposing clinically approved drugs for GBM provides a way to identify potential compounds that could be progressed to the clinic faster than experimental drugs.

Previous unpublished research within our group employed various omics technologies, including transcriptomics, proteomics, and metabolomics, to identify potential drug targets within the invasive margin. This project aimed to identify potential drug candidates for repurposing against these targets through filtering down clinically approved drugs obtained from *in silico* molecular docking. These successful drug candidates were tested in a range of *in vitro* drug assays to determine their cytotoxicity against GBM invasive margin cell lines. The final part of this project aimed to identify potential drug targets within GBM invasive margin tumour tissue samples. Unfortunately, potential drug targets could not be identified in primary tissue due to experimental issues, but it paves the way for future studies to explore this using the pipeline established in this project.

4.1 Identifying Cytotoxic Drug Candidates Against the GBM Invasive Margin

This project aimed to identify potential repurposed drug candidates against omics-derived drug targets in the invasive margin, through the evaluation of potential candidates identified by *in silico* molecular docking. This aim was satisfied as six drug candidates were identified through this process. Idelalisib, domperidone and carvedilol were identified against transcriptomic-derived targets, and nilotinib, darifenacin and

lurasidone were identified against proteomic-derived targets. These drug candidates were then tested using *in vitro* drug assays to assess the cytotoxic potential of these drugs against GBM invasive margin cells. GIN cell lines were chosen as these are primary cell lines derived from the invasive margin of GBM patients. The six identified drug candidates were tested alongside three drugs that target metabolomic-derived targets. Metabolomic-derived targets were selected from the glycerophospholipid pathway, which was observed to be dysregulated in GBM invasive margin cell lines compared to the corresponding GBM tumour core cell lines. Literature searches were conducted to identify therapeutics that target each metabolic enzyme in this pathway. The drugs were then selected based on the cytotoxic effects observed in other cancers and GBM cell lines in the literature. This process identified RSM932A, U-73122, and MAFP. Out of these nine drugs, six conferred cytotoxic effects within GIN cell lines, consistent with the hypothesis.

4.1.1 Domperidone and Carvedilol are Cytotoxic Against the GBM Invasive Margin

The three drug candidates that were selected for the transcriptomic-derived targets were idelalisib, domperidone and carvedilol. As there was no previous literature concerning the use of these drugs in GBM cells, their effect and IC₅₀ needed to be established. Domperidone and carvedilol both induced varying degrees of cell death in all three cell lines, with carvedilol being the most potent. GBM exhibits significant inter-patient heterogeneity, which may account for the variability observed. Surprisingly, idelalisib did not cause complete cell death at the highest concentration, even though it is a chemotherapeutic approved for the treatment of certain blood cancers. Further testing at higher concentrations was not carried out, as the need for higher *in vitro* concentrations would translate to proportionally higher concentrations required for *in vivo* testing in the clinical setting. The higher dosing required would increase the risk of adverse effects and systemic toxicity.

Domperidone and carvedilol were then tested in 3D spheroids, where the IC₅₀ for each drug in each cell line increased. This is likely due to the drugs having to penetrate multiple cell layers to reach the core of the spheroid. 3D spheroids are a more accurate

representation of the disease, as the drug would have to penetrate not only the cell layers where tumour cells have clumped together, but also the healthy brain tissue to reach these cells. Therefore, the IC₅₀ produced when testing in 3D models is likely more accurate and will better inform dosing when these drugs are used in future *in vivo* studies.

At the time of drug selection, there was no existing literature surrounding the use of these drugs in GBM. Since then, domperidone has been tested in one GBM cell line, A172. In our study, the IC₅₀ values observed were between 55 to 65 µM against the GIN lines, indicating cytotoxic effects, whereas no cytotoxic effects were observed up to 100 µM against the A172 cell line (Moura, Gouveia and Vale, 2025). The A172 cell line is a conventional GBM cell line that is likely derived from the tumour core, not the invasive margin, like GIN cell lines are. Our results suggest that domperidone may specifically target invasive margin GBM cells, further supporting the rationale that the invasive margin is molecularly different to the tumour core and better represents residual disease post-surgery.

Both domperidone and carvedilol are predicted to bind to the ZNF185 gene. To our knowledge, this is the first study targeting the ZNF185 gene in the context of GBM and other brain tumours. ZNF185 has been implicated in various other cancers, but for distinct biological processes. In pancreatic cancer, increased expression is linked to treatment resistance, survival, and proliferation (Fang *et al.*, 2020). However, one study describes ZNF185 as a p53 target gene, and the authors found that depletion of this gene in different epithelial tumours contributes to cancer cell migration (Smirnov *et al.*, 2018). While the role of ZNF185 varies in different cancers, our study suggests that ZNF185 may contribute to the malignant and invasive phenotype of GBM cells, as targeting it with therapeutics impaired proliferation and triggered cell death. While domperidone was predicted most likely to bind to ZNF185, it was also predicted to potentially bind to MRGPRX3 and RENBP. These two genes have also not been investigated in GBM, nor have they been widely studied within any cancer, and therefore, their role in cancer progression is unknown. As idelalisib did not cause complete cell death, this may suggest that the RENBP gene does not have a role in GBM cell proliferation or survival.

4.1.2 Nilotinib and Darifenacin are Cytotoxic Against the GBM Invasive Margin

The three drug candidates selected against the proteomic-derived targets were nilotinib, darifenacin and lurasidone. Nilotinib inhibited cell growth in all three GIN cell lines with varying IC_{50} values, which could be explained by the high degree of heterogeneity observed within GBM. Nilotinib is the most potent therapeutic of the three, making it the most promising candidate for *in vivo* studies. An average peak plasma concentration of nilotinib when 400 mg is given twice a day is 1,595 ng/mL (Tian *et al.*, 2018). Due to the low micromolar concentrations required to reduce cell viability in GIN cell lines, nilotinib could likely reach a therapeutic concentration for GBM treatment clinically. Darifenacin also inhibited cell growth in all three GIN cell lines with varying IC_{50} values; however, it was not as potent as nilotinib. The highest approved dose of darifenacin is 15 mg, which is unlikely to reach the therapeutic concentration needed to trigger cell death in GBM invasive margin cells clinically (Skerjanec, 2006). To overcome this, further tests and clinical trials would be needed to assess the safety of higher doses in patients, or where different administration methods could be investigated, such as localised application into the brain cavity during surgery. Lurasidone did not cause complete cell death at the highest concentration tested. Further tests at higher concentrations were not conducted, as higher concentrations needed in 2D monolayers would translate to higher concentrations needed *in vivo*, increasing the risk of adverse systemic effects when administered to patients.

Nilotinib is predicted to bind to LOXL1, which is overexpressed in GBM and associated with a poor prognosis. Nilotinib showed cytotoxic effects at very low concentrations against GIN cell lines with IC_{50} values ranging from 3.5 μ M to 13.9 μ M. This suggests the protein LOXL1 may contribute to cell survival and progression of the disease. This is consistent with previous research as LOXL1 has been associated with modulating cell proliferation through the Wnt/beta-catenin pathway, and upregulation of anti-apoptotic activity (Yu *et al.*, 2020). Darifenacin targets SH3KBP1, which is highly expressed in GBM and associated with poor prognosis. These findings suggest that SH3KBP contributes to disease progression and that inhibiting this protein impairs proliferation and triggers cell death. This is consistent with previous research detailing that SH3KBP1 plays a role in regulating oncogenic EGFR signalling, contributing to

cell proliferation, migration and tumour growth (Song *et al.*, 2021). Lurasidone was predicted to bind to A2M, and no cytotoxic effect was observed in our study. A2M has not been studied in the context of GBM, but has been studied in astrocytoma, which is a type of brain tumour. Within astrocytomas, A2M is upregulated and can interact with low-density lipoprotein receptor-related protein 1, which inhibits cell proliferation, migration and invasion (Huang *et al.*, 2005; Lindner *et al.*, 2010). If the function of A2M is similar in GBM to the function in astrocytoma, it may naturally suppress cell proliferation, so inhibiting it would have no effect. Therefore, further research into the function of A2M in GBM may reveal that it has tumour suppressive effects, similar to what is observed in astrocytomas.

4.1.3 RSM932A and U-73122 Exhibited Cytotoxic but Not Anti-Invasive Effects Against GBM Invasive Margin Cells

RSM932A and U-73122 exhibited dose-dependent cytotoxic effects against GIN28 and GIN31 cell lines when used to target the glycerophospholipid pathway (identified through metabolomic profiling). RSM932A was the most potent drug with a very low micromolar IC₅₀ concentration. This is consistent with a study using RSM932A in a panel of various cancer cell lines (Lacal and Campos, 2015). This study observed a lower IC₅₀ value when RSM932A was tested in U87 cells, which is a GBM cell line derived from the tumour core, relative to what was observed in our study. Testing this drug in GIN cells produced higher IC₅₀ values, further highlighting that therapeutics should be tested against GBM invasive margin cells and models to gain a better understanding of how they work in this population, and for greater clinical relevance. U-73122 also produced low micromolar IC₅₀ concentrations, but it was not as potent as RSM932A. The IC₅₀ values obtained for U-73122 in GIN cell lines are similar to the most effective concentration of U-73122 that causes minimal damage to astrocytes when used in the T98G GBM cell line (Zajac *et al.*, 2025). This could suggest that U-73122 may be a promising candidate for future work, as the risk of toxicity to healthy brain cells is low. However, MAFP exhibited no cytotoxicity against either cell line. This is consistent with the literature, as it has been observed that MAFP can successfully inhibit serine hydroxylase activity in healthy brain tissue but is less effective in GBM tissue (Aaltonen *et al.*, 2020).

As GBM is highly heterogeneous, using multiple therapies in combination may increase the chances of eradicating the malignant cells left behind after surgery. Therefore, RSM932A and U-73122 were tested in synergy as they target two different metabolic enzymes associated with the glycerophospholipid pathway. RSM932A targets choline kinase, and U-73122 targets phospholipase C. They were tested in GIN28 and GIN31 cell lines, where they exhibited distinct potencies, further confirming the high degree of heterogeneity between GBM tumours. When testing these drugs in synergy, they were more potent in GIN28 cells, consistent with the results obtained when testing each drug individually in the cell lines. The global synergy scores observed in both the GIN28 and GIN31 cell lines indicated a potential additive effect of both RSM932A and U-73122. One antagonistic value was observed in the GIN28 synergy assay, -12.02, at the lowest concentration of each drug combined. This only resulted in 38% cell death, so these concentrations would not be used to achieve maximal cell death. In the GIN31 cell line, some values were almost synergistic with synergy values of 8.32 and 8.08, indicating that when these drugs are combined, they display better efficacy in GIN31 cells. Overall, there is no synergistic effect when these drugs are combined; there is only an additive effect, suggesting these drugs are more effective when administered together.

The effects of RSM932A and U-73122 on invasion were tested in the GIN28 and GIN31 cell lines, and no significant reduction in invasion was observed. As these drugs are cytotoxic, cell viability for each condition was measured to determine if there may have been a reduction in cell numbers. A large reduction in cell viability may translate to a reduction in invasion, or cells may be able to invade more easily as there is less competition. When the data obtained from the invasion assay was normalised to cell death, there was no reduction in invasion observed in the GIN28 cells and in three conditions for the GIN31 cells. As the IC_{25} and IC_{50} concentrations were obtained from a 72-hour viability assay, the reduction in cell viability after 24 hours was unexpected. When considering the cytotoxic effects of these drugs, an overall increase in invasion was observed, particularly at the IC_{50} concentrations of the drugs when applied individually. A ratio value of more than one indicates increased invasion. However, within the GIN31 cell lines, three conditions showed a reduction in invasion, with a ratio value of less than one. As most conditions experienced an increase in invasion, these findings may suggest that RSM932A and U-73122 do not have anti-invasion

properties. This result is particularly surprising for U-73122, as previous studies have shown that the drug reduced invasion in patient-derived GBM cell lines and U87 GBM cell lines (Yang *et al.*, 2023). The data from the 24-hour viability assay imply that the drug concentrations used in the invasion assay may have been too high, such that the cytotoxic effects masked any potential anti-invasion activity. Nevertheless, these findings may indicate that the glycerophospholipid pathway does not play a significant role in cell invasion at the GBM invasive margin.

4.1.4 Future Work

Future work for domperidone and carvedilol should involve validating the specificity of their target. Domperidone and carvedilol exert their cytotoxic effect through inhibition of the protein encoded by the ZNF185 gene. To achieve this, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) could be used to measure any change in the messenger RNA levels of ZNF185 caused by the drugs. To validate this further, a Western blot can be performed to investigate if these changes at the transcriptional level result in changes at the protein level. Domperidone and carvedilol are also good candidates to test in more advanced clinical models. For example, to determine whether domperidone and carvedilol exhibit selective cytotoxicity against GBM invasive margin cells. To test this, co-culture models incorporating human astrocytes could be used to predict if these drugs will be cytotoxic to human brain cells. These drugs can also be tested in Matrigel models using 3D spheroids of GIN cell lines. This recapitulates the ECM that would be present *in vivo* and will allow investigation of cellular behaviour and drug penetration, which will help prepare the drugs for *in vivo* tests, such as in patient-derived orthotopic xenografts.

As nilotinib and darifenacin were cytotoxic against 2D monolayers and there was no time to test them in 3D models, future work could involve testing them in more advanced models. It is expected that nilotinib and darifenacin would exhibit cytotoxic effects in 3D models. This can be tested against the same three GIN cell lines in 3D spheroids at the same concentrations. This will provide more accurate IC₅₀ values, as they are a more accurate representation of the tumour microenvironment. Similar to domperidone and carvedilol, Western blots can be used to confirm that nilotinib exerts its effects through LOXL1 and darifenacin exerts its effects through SH3KBP1. As

nilotinib and darifenacin target different proteins, when combined, a synergistic cytotoxic effect may be observed. To test this, various concentrations around the IC₅₀ of each drug could be used in combination, and cell viability could be measured using a Presto Blue™ assay.

For RSM932A and U-73122, future work could include repeating the invasion assay. Reducing the concentrations used for each condition in the invasion assay should minimise the cytotoxic effect of each drug and allow the potential anti-invasion effect to be decoupled. To test this, the same experiment should be conducted, but IC₅, IC₁₀ or IC₂₀ concentrations for each drug should be used to minimise the potential cell death. As both drugs are very potent in 2D GIN cell lines, testing them in 3D spheroids would provide more accurate results on the potency of both drugs.

4.1.5 Limitations

When testing nilotinib in GIN8 cells, only two biological replicates were able to be obtained due to time constraints. As there are only two repeats, the result is not reliable as there are limited degrees of freedom. A further repeat for GIN8 should be obtained to establish $n=3$ to ensure the data is more reliable and robust. As GBMs are highly heterogeneous, it is likely that the three cell lines used do not fully capture this heterogeneity. Even though the results obtained for the same drug from each cell were similar, it cannot be assumed that the drug would invoke similar responses in additional primary cell lines derived from the invasive margin. To combat this limitation, a larger panel of cell lines could be used. However, this was not possible within the scope of this project due to the time constraints and availability of these cell lines. Even though the cell lines used are primary cell lines, there is no tumour microenvironment which can influence cellular characteristics. When cells are grown in a 2D monolayer, they lose the interaction with the ECM that would naturally occur in the brain, which can cause changes in adhesion, survival, and migration. They also lose cell-cell interactions. Within the brain, GBM cells interact with healthy brain cells, such as neurons, but also immune cells recruited to the tumour. Various studies have shown that tissue-cultured cells display molecular differences to actual tumour tissue (Vogel *et al.*, 2005; Shen *et al.*, 2019; Park *et al.*, 2024). While using more complicated disease models (such as 3D models, Matrigel, and patient-derived xenografts) can

recapitulate more of the tumour microenvironment, it is a difficult limitation to overcome.

4.2 Identifying Drug Targets in GBM Invasive Margin Tissue Samples

To the best of our knowledge, this is the first study to attempt to profile protein expression of the 5-ALA positive population of cells in the GBM invasive margin. This study aimed to identify potential drug targets in the form of ubiquitous and differentially expressed proteins in the 5-ALA positive invasive margin. Unfortunately, the aims for this part of the project were unable to be met due to the presence of a contaminant in all the samples, which was introduced during the protein extraction process. Therefore, a comparison between protein expression in the 5-ALA positive population and the 5-ALA negative population was unable to be made. However, some proteomic data was able to be obtained from remaining samples leftover from FACS that had not yet been through the protein extraction stage.

As two 5-ALA negative samples (i.e., non-disease cells) were used, proteins present in these two samples could be removed from the list to leave proteins only present in the mixed sample. Theoretically, these remaining proteins could be found within the 5-ALA positive population, as they are absent in the 5-ALA negative populations. From this, the top ten abundant proteins were identified. Many of these proteins have already been identified within GBM, supporting the suggestion that they are from the malignant cell population. HK2 is crucial for the Warburg effect within cancer, and increased expression of HK2 has been observed within GBM and correlates with worse overall survival (Wolf *et al.*, 2011). Overexpression of HK3 is particularly seen within the TCGA mesenchymal subtype and correlates with poorer prognosis (Li *et al.*, 2023). The YWHA protein family can interact with a diverse array of proteins and is involved in vital processes such as cell death, cell cycle regulation, metabolism, and signal transduction, making these ideal cancer therapeutic targets (Morrison, 2009). YWHAB and YWHAZ are overexpressed in GBM and have roles in proliferation, cell survival, migration, and invasion of GBM cells (Park *et al.*, 2012; Yang *et al.*, 2019; Gallo *et al.*, 2023). PGK1 is upregulated in GBM and is involved in tumourgenesis, glycolysis, angiogenesis and cell migration and invasion (Irshad *et al.*, 2015). The IGHG family are crucial to produce immunoglobulins and has been implicated in

various cancers, including GBM, which displays upregulated IGHG1 and IGHG2 (Serão *et al.*, 2011; Wu *et al.*, 2025). Upregulation of F13A1 in GBM is associated with a poor prognosis and an immunosuppressive phenotype (Liu *et al.*, 2025). Many of the actin isoforms are upregulated in GBM. ACTG1 is highly expressed within GBM and plays a role in cell survival and proliferation (Wanibuchi *et al.*, 2018; Zhou *et al.*, 2020; Comba *et al.*, 2022). These are all potential ideal drug targets as many play a role in disease progression and have been observed in previous studies. As the findings of this study are consistent with the literature, this method can successfully identify differentially expressed proteins which could act as therapeutic targets.

However, many of these proteins have not been observed previously in GBM but play a role in various other forms of cancer. DST is involved in cell-cell adhesion and has been observed in many cancers. It is overexpressed in melanoma, supporting malignant cell growth and is associated with poor survival (Leick *et al.*, 2019). The POTE family of proteins are widely expressed in normal tissues but are upregulated within some cancers. POTEE is associated with poor survival in colorectal, pancreatic and lung cancer, while POTEF is involved in the initiation and progression of prostate cancer (Leick *et al.*, 2019; Li *et al.*, 2020). HSD17B12 is overexpressed in ovarian, breast, colorectal, and squamous cell cancers, and is associated with poorer survival (Szajnik *et al.*, 2012; Lin *et al.*, 2020). MNDA has roles in innate immunity, inflammation, and apoptosis, and is involved in various forms of cancer with different expression levels. It is upregulated in gastric cancer cells and chronic lymphocytic leukaemia, but downregulated in lung cancer (Bottardi *et al.*, 2020; Tang *et al.*, 2022; Zhao *et al.*, 2024). CORO2B has been implicated in cancer, such as high expression in oesophageal squamous cell carcinoma, which correlates with improved survival (Zhang *et al.*, 2020). These proteins are potential novel drug targets that have not previously been identified in GBM, to our knowledge. As many of these are linked to prognosis or various cancer hallmarks, they are promising candidates to investigate further. This will further the understanding of the roles these proteins play in GBM disease progression, identifying key processes that could be therapeutically targeted.

When comparing the list of proteins identified from tumour tissue samples in this study to the proteins identified in GIN cell lines in the previous study, the results were unexpected. The GIN cell lines used in the previous study are primary cell lines derived

from the same 5-ALA positive invasive margin as the tissue samples, albeit the cell lines and tissue were derived from different patients. Because these two studies focused on cells and tissue from the same tumour area, it was hypothesised that the proteins detected in the cell lines would be detected in the tissue samples. However, that was not the case as only six out of the seventeen proteins were detected in this study, leading to the rejection of the hypothesis. Three of these proteins, APOL2, CD58 and EDIL3 were detected in the mixed sample and not the two 5-ALA negative samples, suggesting their presence may be due to the 5-ALA positive tumour cell population. CD58 is the only protein that has previously been linked to GBM. High expression is associated with poorer prognosis, increased stemness and immunosuppression within GBM (Wu, Zhan and Jiang, 2024). EDIL3 has been reported in various cancers, both up- and down-regulated, and has critical roles in tumourigenesis, angiogenesis, migration and invasion (Ke *et al.*, 2024). These three proteins could represent selective drug targets against the invasive margin to prevent recurrence. A2M and the RAC family of proteins were detected in all three samples, but the expression levels cannot be compared as the LC-MS samples contained different protein concentrations.

Surprisingly, PZP was detected in the 5-ALA negative samples and not in the mixed sample, even though it was detected in the GIN cell lines. Cells that are grown in culture have a different microenvironment from what would be present in the brain. Various studies have investigated the molecular differences between GBM cells that have been tissue cultured and primary GBM tissue samples. A previous study identified a gain of around 160 proteins and a loss of around 60 proteins in cultured GBM cells compared to GBM tissue samples (Vogel *et al.*, 2005). Another study found significant dysregulation of genes between GBM tumour tissue and cell lines, with genes related to L1CAM interactions and synaptic signalling upregulated in tissue (Batchu *et al.*, 2023). When GBM cells are grown in culture, cell-cell communication with somatic or cancer-associated cells, such as neurons and immune cells, is lost, which can trigger molecular changes within the cell. It has also been found that genes involved in invasiveness, such as STAT3, are downregulated in GBM cell lines, whereas genes involved in cell proliferation are upregulated (Park *et al.*, 2024). This poses a major problem as invasiveness is a critical factor in the recurrence of GBM, yet the molecular characteristics cannot be conserved in tissue culture. This may

explain why proteins observed in GIN cell lines are absent in tumour tissue samples, and proteins only present in tissue samples are absent in cell lines.

4.2.1 Future work

As the aims and objectives of this part of the project could not be met, this pipeline should be repeated using at least three patient samples that have been sorted into 5-ALA positive and negative populations. Ubiquitous, differentially expressed proteins in the 5-ALA positive cell population would act as ideal novel drug targets in the GBM invasive margin. To identify these protein targets, LC-MS data would be analysed using Amica to identify proteins that are up- or down-regulated in the 5-ALA positive population compared to the 5-ALA negative population. Therapeutics identified against these protein targets could be cytotoxic, causing cell death in the invasive margin population. Molecular docking approaches would be used to identify drugs that could bind to these proteins. These drugs would then be tested in a panel of GIN cell lines, in 2D and 3D models, to assess their cytotoxicity.

4.2.2 Limitations

There are several limitations to the uncontaminated data obtained in this part of the project. As the patient 5 sample had not undergone FACS, it cannot be certain if the proteins that were identified as present in the patient 5 sample, but absent in the 5-ALA negative samples, are due to the 5-ALA positive population. Another limitation is that the concentration of protein in each sample was different for LC-MS, meaning the data can only be considered qualitatively and not quantitatively. This means that in the high protein mixed sample, proteins at a low abundance may have been blocked by highly abundant protein signals. Proteins in the 5-ALA negative low concentration samples may contain proteins for which the signal did not reach the detection threshold, so they were missed. Using uniform concentrations would increase the reliability of this data. Another limitation is the number of repeats. There was only one mixed sample, and two 5-ALA negative samples, so reliable conclusions about this data cannot be made.

4.3 Conclusion

GBM is a highly heterogeneous disease, with most research focusing on the tumour core rather than the clinically relevant invasive margin. This project aimed to identify potential therapeutics against omics-derived targets identified in the GBM invasive margin and to assess their cytotoxicity. This aim was successfully achieved as nine potential therapeutics were identified, six of which proved to be cytotoxic against GBM invasive margin cell lines. The nine potential candidates identified were idelalisib, domperidone, carvedilol, nilotinib, darifenacin, lurasidone, RSM9342A, U-73122 and MAFP. The results show six of these exhibited cytotoxic effects: domperidone, carvedilol, nilotinib, darifenacin, RSM932A, and U-73122. The second aim was to identify potential drug targets in the form of ubiquitous, differentially expressed proteins in GBM invasive margin tissue samples. Unfortunately, this aim could not be fulfilled due to contamination within the samples, which obscured the peaks of each detected protein, rendering the data unusable. However, the contaminant and its introduction into the samples was identified. Despite this, this project has established a pipeline for separating 5-ALA fluorescent tissue in the invasive margin and extracting proteins to identify selective therapeutic targets in future studies. This study has highlighted the potential of using omics approaches to identify potential drug targets and the importance of targeting the invasive margin region in GBM.

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