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Master by Research Title:

**Investigating 3D – Printed Models for Developing Less
Invasive Therapeutic Approaches in Glioblastoma**

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Abbreviation	Definition
AKT	Protein Kinase B
AMPK	AMP-Activated Protein Kinase
ANOVA	Analysis of Variance
APAF1	Apoptotic Protease Activating Factor 1
ATP	Adenosine Triphosphate
AXIN1	Axis Inhibition Protein 1
β-AC	β-Amino Carbonyl
3DP	Three-Dimensional Printing
BBB	Blood-Brain Barrier
BCL2	B-Cell Lymphoma 2
BCL2L1	BCL2-Like Protein 1
BCL2L2	BCL2-Like Protein 2
BTRC	Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase
CAIX	Carbonic Anhydrase IX
CASP8	Caspase 8
CcND1	Cyclin D1
CDK6	Cyclin-Dependent Kinase 6
CNS	Central Nervous System
COL1A1	Collagen Type I Alpha 1 Chain
CORO1C	Coronin-1C
CT	Computed Tomography
DGCR8	DiGeorge Syndrome Critical Region Gene 8
Dicer	Double-Stranded RNA-Specific Endoribonuclease Dicer
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DVL	Dishevelled Protein
E2F	E2F Transcription Factor
ELK1	ETS Like-1 Protein
EMT	Epithelial-Mesenchymal Transition
EPAS1	Endothelial PAS Domain Protein 1
Exportin-5	Exportin 5
FGF1	Fibroblast Growth Factor 1
FLT1	Fms Related Receptor Tyrosine Kinase 1
FOXO4	Forkhead Box O4
GB	Glioblastoma
HDAC	Histone Deacetylase
HIF-1α	Hypoxia-Inducible Factor 1 Alpha
Hippo	Hippo Signalling Pathway
HPMC	Hydroxypropyl Methylcellulose
IC50	Half-Maximal Inhibitory Concentration
IDH	Isocitrate Dehydrogenase
LARP1	La-Related Protein 1
LATS1	Large Tumour Suppressor Kinase 1
MAGI1	Membrane Associated Guanylate Kinase, WW and PDZ Domain Containing 1
MAPK	Mitogen-Activated Protein Kinase
MAP2K4	Mitogen-Activated Protein Kinase Kinase 4
MAP3K	Mitogen-Activated Protein Kinase Kinase Kinase
MMP	Matrix Metalloproteinase
miRNA	MicroRNA
MRI	Magnetic Resonance Imaging
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NaCMC	Sodium Carboxymethylcellulose
PAK	p21-Activated Kinase
PI3K	Phosphoinositide 3-Kinase
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIK3R1	Phosphoinositide-3-Kinase Regulatory Subunit 1
pre-miRNA	Precursor MicroRNA
pri-miRNA	Primary MicroRNA
PTEN	Phosphatase and Tensin Homolog
RB1	Retinoblastoma 1
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SHG-8	β-(4-Bromophenyl)-1-Phenyl-3-(Phenylamino)Propan-1-One
SIRT1	Sirtuin 1
SMAD	Small Mothers Against Decapentaplegic
sRNA	Small RNA
TGA	Thermogravimetric Analysis
TGF-β	Transforming Growth Factor Beta
TGFBR	Transforming Growth Factor Beta Receptor
TMZ	Temozolomide
TRBP	TAR RNA-Binding Protein
TRIM33	Tripartite Motif Containing 33
U87MG	Human Glioblastoma Cell Line U87MG
U251MG	Human Glioblastoma Cell Line U251MG
UK	United Kingdom
VEGFA	Vascular Endothelial Growth Factor A
Wnt	Wingless/Integrated Signalling Pathway
YAP1	Yes-Associated Protein 1

Abstract

Glioblastoma is the most aggressive and common primary brain tumour in adults, characterized by rapid growth, extensive invasiveness, and poor overall prognosis. Its heterogeneous nature and tendency to infiltrate surrounding brain tissue make accurate diagnosis challenging, often delaying detection until the disease reaches advanced stages. Current treatment options, including surgical resection, radiotherapy, and chemotherapy, provide limited improvement in patient survival and are frequently associated with significant systemic toxicity, drug resistance, and recurrence. These limitations underscore the urgent need for novel therapeutic agents and innovative drug delivery strategies that can enhance efficacy while minimizing adverse effects. In this study, *in vitro* assays were performed to evaluate the anti-glioblastoma activity of the in-house synthesized β -amino carbonyl compound SHG-8 delivered through a 3D-printed hydrogel patch system in U87MG glioblastoma cells. The SHG-8-loaded patch demonstrated dose-dependent cytotoxicity and significant inhibition of colony formation indicating anti-proliferative effects against glioblastoma cells. The enhanced therapeutic activity observed with the 3D-printed patch likely reflects the benefits of localized and sustained drug delivery together with hydrogel-mediated modulation of the tumour microenvironment. These findings highlight SHG-8 as a promising therapeutic candidate for glioblastoma and emphasize the importance of delivery strategy in optimizing drug potency. The integration of advanced biomaterials, such as 3D-printed hydrogels, provides a strategy to improve localized drug exposure, reduce systemic toxicity, and potentially overcome limitations of conventional therapies.

1. Introduction

Brain and central nervous system (CNS) tumours are common across different age groups. They are one of the most frequent cancers in adolescents and young adults and are also seen in older adults. Each year, many brain and CNS tumour cases are diagnosed worldwide. In the United Kingdom, many people are diagnosed annually, and a significant number of deaths are reported. In 2022, approximately 322,000 new cases of brain and central nervous system tumors were reported worldwide (Filho et al., 2025). Although survival over shorter periods is relatively high, long-term survival remains low because of rapid disease progression and treatment failure (McFaline-Figueroa et al., 2018). In adults, most primary brain tumours are diffuse gliomas. These include types such as isocitrate dehydrogenase (IDH)-mutant astrocytoma, oligodendroglioma, and IDH-wildtype glioblastoma (Han et al., 2020). In the past, only grade 1 and grade 2 tumours were considered low-grade gliomas. However, some grade 3 IDH-mutant gliomas are now also included because patients with these tumours tend to have better survival outcomes (Bent et al., 2023)

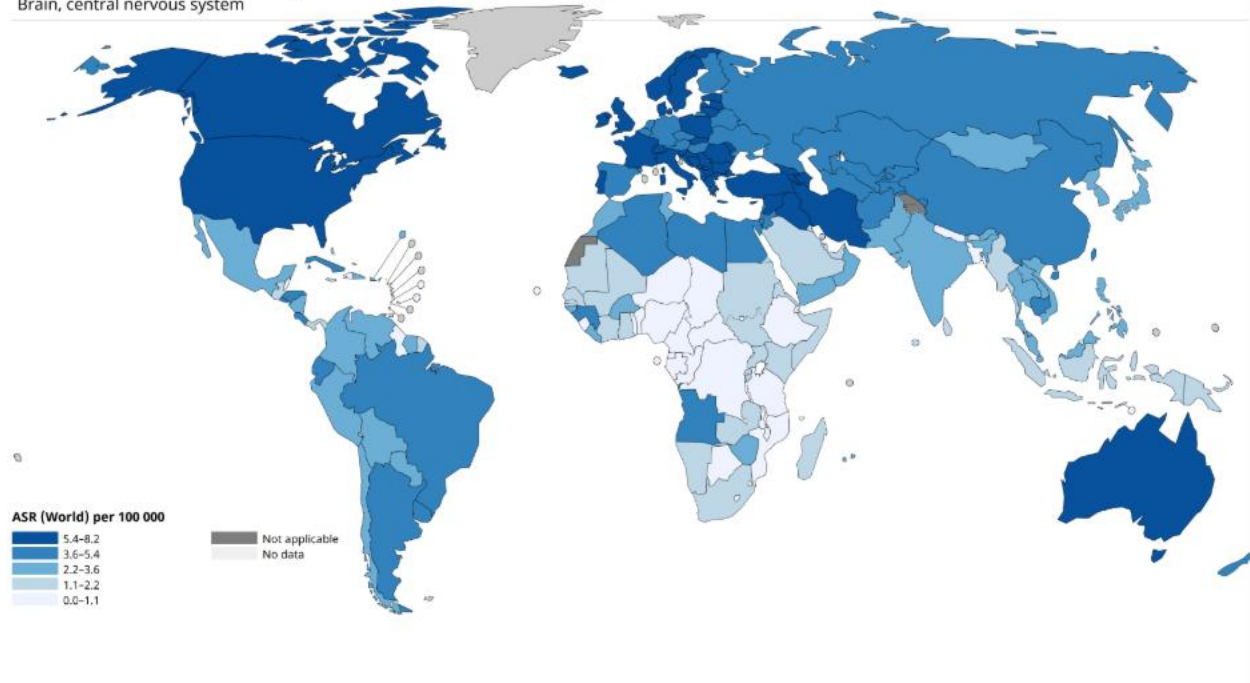
1.1 Glioblastoma

Glioblastoma (GB) represents the most aggressive and rapidly progressing form of primary brain tumour and is classified as a grade IV astrocytoma. It originates from astrocytes, a subtype of glial cells responsible for maintaining neuronal support and homeostasis within the CNS and it most commonly arises in frontal or temporal lobes (Davis et al., 2016). GB is characterized by marked heterogeneity at both the cellular and molecular levels, which contributes to its biological complexity and therapeutic resistance (Grochans et al., 2022). Key pathological features include uncontrolled cellular proliferation, diffuse infiltration into adjacent brain tissue, extensive necrosis, and pronounced angiogenesis involving structurally abnormal vasculature. These characteristics collectively underpin the tumour's highly invasive behavior and its limited responsiveness to conventional treatment strategies (Grochans et al., 2022). At the molecular level, GB is driven by a range of genetic and epigenetic alterations that disrupt key cellular processes, including cell cycle regulation, DNA repair, and apoptosis. These changes facilitate uncontrolled proliferation, tumour progression, and resistance to therapy (Singh et al., 2025). The tumour microenvironment also plays a critical role in disease progression by promoting immune evasion and supporting tumour survival. Molecular biomarkers are increasingly utilized in the classification and management of glioblastoma, providing valuable prognostic information, and identifying potential therapeutic targets. Advances in molecular and genomic research are contributing to the development of more personalized treatment approaches, although translating these findings into effective clinical therapies remains a significant challenge (Singh et al., 2025).

1.2 Epidemiology

Glioblastoma is the most frequently occurring malignant primary brain tumour in adults and accounts for a substantial proportion of central nervous system cancers. It remains a major clinical concern due to its high mortality rate and the limited efficacy of current therapeutic interventions (Gillepsie et al., 2022). Both within the United Kingdom and globally, glioblastoma contributes significantly to the overall burden of brain tumours (Figure 1). Glioblastoma has an overall survival of about 6 to 17 months and is responsible for around 3,350 new diagnoses in the UK, while posing a significant burden because of its poor prognosis (Gillepsie et al., 2022). Despite advances in diagnostic imaging and treatment approaches, survival outcomes have shown minimal improvement over time. The impact of malignancy extends beyond clinical outcomes, affecting patients' quality of life, and placing considerable strain on healthcare systems and support networks.

Age-Standardized Rate (World) per 100 000, Incidence, Both sexes, in 2022
Brain, central nervous system



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International Agency
for Research on Cancer
World Health
Organization

Figure 1: Global map of age-standardized incidence rates (per 100,000 population) for brain and central nervous system cancers in 2022. Darker shades indicate higher incidence rates, with the highest levels observed in regions such as North America, Europe, and Australia. Intermediate rates are seen across parts of South America, Asia, and North Africa, while the lowest incidence rates are reported in many regions of Sub-Saharan Africa and parts of Southeast Asia. The map highlights clear geographic disparities in brain cancer incidence, which may reflect differences in population demographics, environmental factors, diagnostic capacity, and healthcare systems (World Health Organization).

1.3 Symptoms and Risk Factors

The clinical presentation of glioblastoma is variable and largely dependent on tumour location, size, and rate of progression. Common symptoms include persistent headaches, nausea, vomiting, and seizures. Patients may also present a range of neurological impairments, such as cognitive decline, memory disturbances, personality changes, and deficits in speech, vision, or motor function. Due to the tumour's aggressive growth and infiltrative nature, these symptoms often progress rapidly (IJerman-Korevaar et al., 2018). In later stages, increased intracranial pressure and involvement of critical brain regions may result in severe neurological compromise, including loss of consciousness and failure of vital physiological functions (IJerman-Korevaar et al., 2018). The etiology of GB is not fully understood, and most cases arise without an identifiable cause. Nevertheless, several risk factors have been associated with its development. Increasing age is one of the most prominent factors, with higher incidence observed in older populations. Exposure to ionizing radiation, particularly during early life, remains the most well-established environmental risk factor. However, given that most cases occur in the absence of these factors, the underlying mechanisms driving tumour initiation remain an area of ongoing investigation (Kanderi et al., 2024).

1.4 Pathology

GB is defined by its highly invasive growth pattern and complex tumour microenvironment (Figure 2). Tumour cells exhibit extensive infiltration into surrounding healthy brain tissue, which significantly limits the effectiveness of surgical intervention. Characteristic features include pronounced cellular atypia, high mitotic activity, microvascular proliferation, and regions of necrosis. Angiogenesis is a key component of tumour progression, driven by hypoxic conditions that stimulate the formation of new, albeit abnormal, blood vessels. Furthermore, glioblastoma cells demonstrate resistance to apoptosis and enhanced ability to adapt to adverse microenvironmental conditions, contributing to disease progression and therapeutic resistance (D'Alessio et al., 2019).

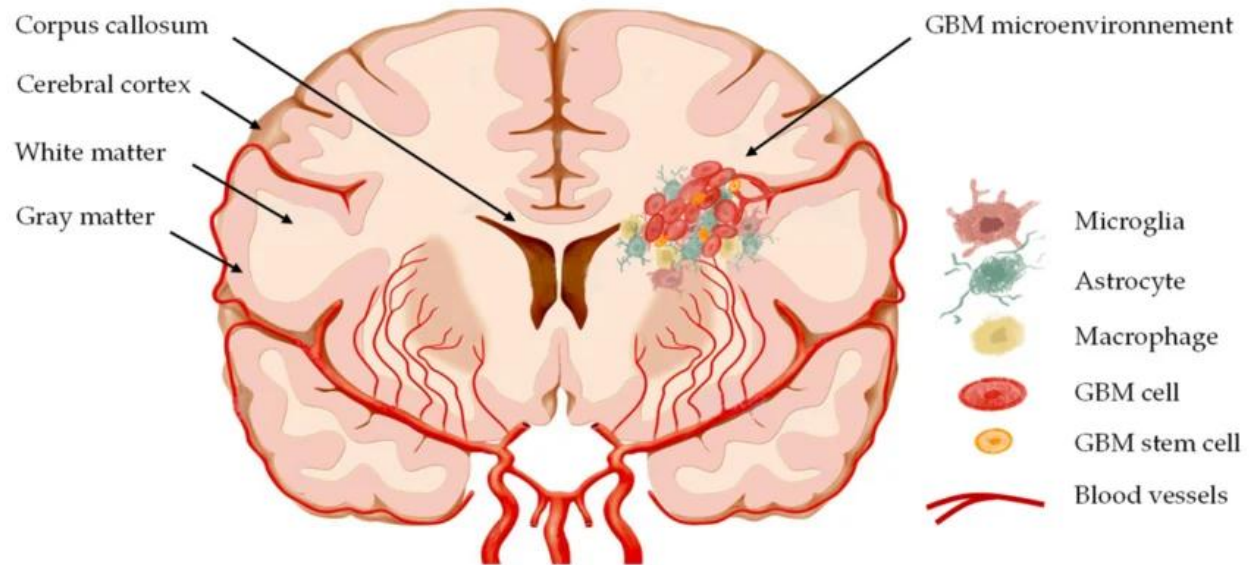


Figure 2: Schematic representation of a cross-section of the human brain highlighting the glioblastoma (GB or GBM) tumour microenvironment. Key anatomical structures, including the cerebral cortex, white matter, gray matter, and corpus callosum, are labelled. The illustration shows the presence of a GB tumour mass within the brain tissue, surrounded by an abnormal vascular network. The tumour microenvironment is composed of various cell types, including GB cells, GB stem cells, astrocytes, microglia, and macrophages, which interact to support tumour growth, invasion, and progression. The diagram also emphasizes the role of blood vessels in supplying nutrients and facilitating tumour expansion (Wiam El Kheir et al., 2022).

1.5 Diagnosis

Diagnosis of GB involves an integrated approach combining clinical assessment, imaging, and histopathological evaluation. Neuroimaging techniques, including computed tomography (CT) and magnetic resonance imaging (MRI), particularly with contrast enhancement, are essential for identifying tumour location and extent (Thenuwara et al., 2023). MRI is especially useful in assessing tumour infiltration and distinguishing neoplastic tissue from surrounding oedema (Figure 3). A definitive diagnosis is established through histological examination of tissue obtained via biopsy or surgical resection. In addition, molecular profiling has become increasingly important in recent years, enabling more precise tumour classification and supporting personalized treatment strategies (Porter et al., 2024).

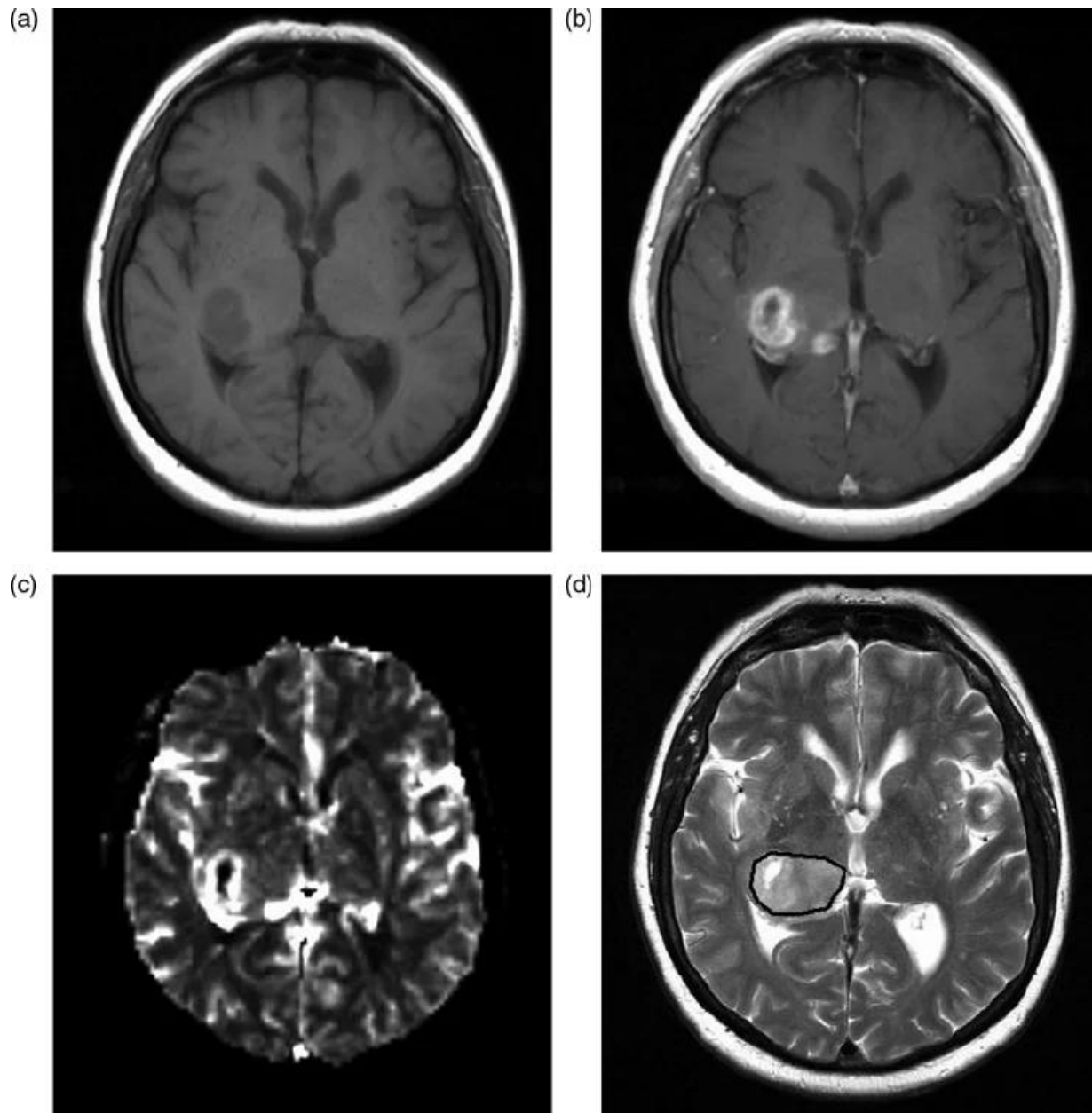


Figure 3: Magnetic resonance imaging (MRI) of a 71-year-old female patient diagnosed with glioblastoma. (a) Axial T1-weighted pre-contrast image demonstrating a hypointense lesion. (b) T1-weighted post-contrast image showing ring-enhancing tumour regions, indicative of blood–brain barrier disruption. (c) Relative cerebral blood volume (rCBV) map highlighting increased perfusion within the tumour, consistent with high vascularity. (d) T2-weighted image illustrating the tumour and surrounding hyperintense oedema, reflecting tumour infiltration and associated tissue changes (Benjamin Garzon et al., 2011).

1.7 Prognosis

The prognosis for patients with GB remains poor despite advances in clinical management (Figure 4). The aggressive nature of the tumour, combined with its resistance to treatment and high rate of recurrence, results in limited survival outcomes. Even with intensive therapeutic intervention, long-term survival is uncommon, and disease progression typically occurs within a relatively short timeframe. These factors highlight the significant challenges associated with improving patient outcomes and underscore the need for continued research in this field (Sipos et al., 2025).

Age-Standardized Rate (World) per 100 000, Incidence and Mortality, Both sexes, in 2022
Brain, central nervous system
Continents

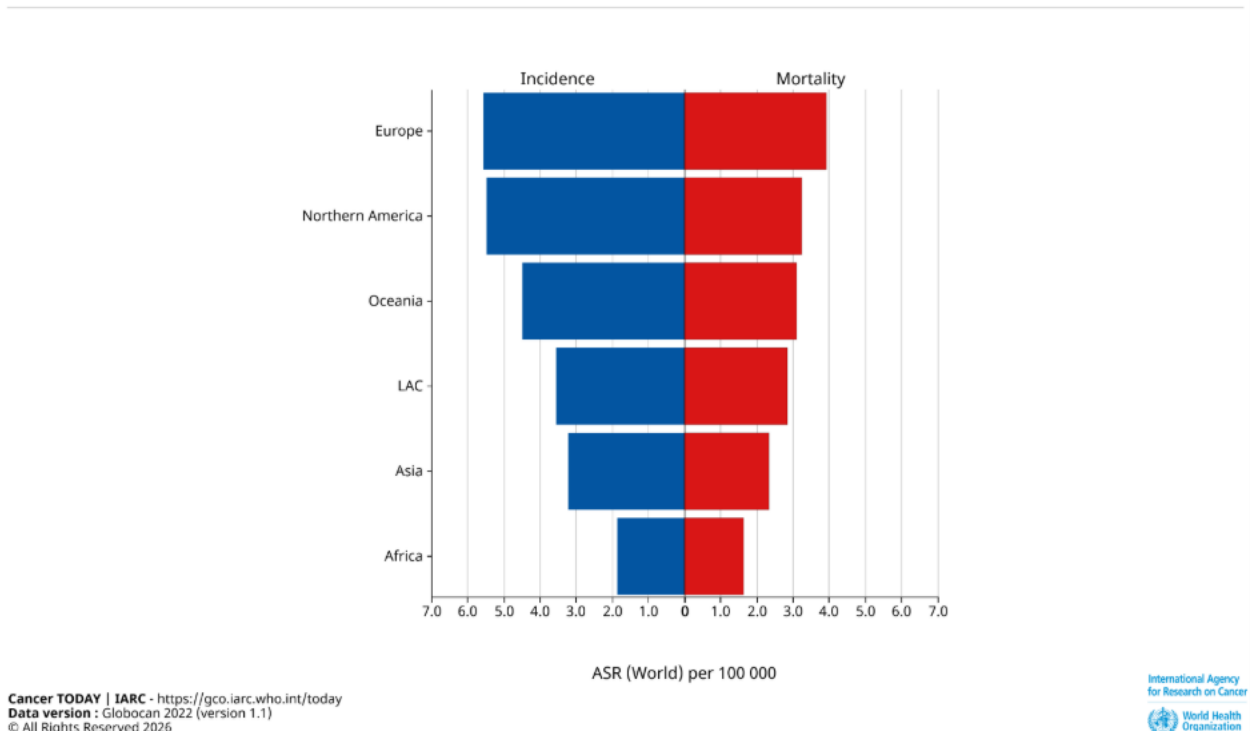


Figure 4: Age-standardized incidence and mortality rates (per 100,000 population) for brain and central nervous system cancers across continents in 2022. The chart shows that Europe and Northern America have the highest incidence rates, followed by Oceania and Latin America and the Caribbean (LAC), while Asia and Africa report lower incidence. Mortality rates follow a similar pattern but are relatively closer across regions, with Africa showing a comparatively higher mortality-to-incidence ratio. Overall, regions with higher incidence also tend to have higher mortality, although disparities suggest differences in diagnosis, healthcare access, and treatment outcomes (World Health Organization).

1.8 Therapy

Management of glioblastoma typically involves a multimodal approach designed to maximize tumour control while preserving neurological function. Standard treatment includes maximal safe surgical resection, followed by radiotherapy and chemotherapy, most commonly with temozolomide (TMZ). However, treatment efficacy remains limited, and recurrence is almost inevitable. Novel therapeutic strategies, including immunotherapies such as pembrolizumab and nivolumab, have triggered safety concerns due to signs of neurotoxicity, and they have not demonstrated any improvement in survival for patients with recurrent GB (Jain et al., 2018). Additionally, the effectiveness of certain chemotherapeutic agents is restricted by systemic toxicity and limited ability to cross the blood–brain barrier. These challenges emphasize the need for more targeted and less toxic therapeutic approaches. Although cisplatin is a highly effective chemotherapy drug, its application in glioblastoma therapy is limited because of its significant systemic toxicity and its inability to adequately cross into brain tumour tissue, even when administered via conventional administration methods (Zhang et al., 2017).

1.11 β -amino Carbonyl (AC) Compounds

Mitochondria play an important role in many cellular processes, including energy production, biosynthesis, redox balance, and apoptosis. In glioblastoma, mitochondrial function is often altered, which contributes to tumour development and progression. Compared to normal cells, tumour mitochondria show higher membrane potential, increased production of reactive oxygen species (ROS), and reduced ability to trigger apoptosis (Hossain et al., 2018). These changes support tumour survival and continuous cell growth. ROS play a key role in glioblastoma progression. At moderate levels, ROS can activate signaling pathways that promote tumour growth. However, when ROS levels become too high, they cause oxidative stress, which damages DNA, proteins, and lipids, and can lead to cell death. Changes in ROS levels can also affect the expression of microRNAs (miRNAs), which regulate genes involved in tumour development, including oncogenes and tumour suppressor genes (Ebrahimi et al., 2020). Because of this, targeting mitochondrial function and ROS balance has become an important area in cancer therapy. β -amino carbonyl (β -AC) compounds are of particular interest as potential therapeutic agents because they can target these mitochondrial abnormalities. These compounds can disrupt the energy balance and redox state of tumour cells, leading to apoptosis. Importantly, this effect is more selective for cancer cells, as normal cells are better able to maintain stable redox conditions. Structurally, β -AC compounds contain a β -amino carbonyl group, which provides stability and improves their bioavailability and pharmacokinetic properties. Due to these characteristics, β -AC compounds are considered promising candidates for cancer treatment, including glioblastoma (Singh et al., 2016). They can target key enzymes and regulatory proteins involved in processes such as cell proliferation, apoptosis, and metastasis. Some β -AC derivatives interfere with DNA replication by targeting topoisomerases, while others inhibit histone deacetylases (HDACs), leading to changes in gene expression that suppress tumour growth. In addition, certain compounds inhibit carbonic anhydrase IX (CAIX), which may help reduce the acidic environment commonly found in tumours (Mardani et al., 2018) (Ceccacci et al., 2016) (Liang et al., 2023).

1.11.1 SHG-8

Recent studies conducted at the University of Hertfordshire have shown that an in-house synthesized β -amino carbonyl compound, b3-(4-bromophenyl)-1-phenyl-3-(phenylamino) propan-1-one (SHG-8), demonstrates strong cytotoxic effects in glioblastoma cell lines, including U251MG and U87MG. The compound was found to significantly reduce cell proliferation and inhibit cell migration, suggesting its potential as an anti-cancer agent. The mechanism underlying these effects is thought to involve disruption of mitochondrial redox balance. β -amino carbonyl compounds such as SHG-8 can alter the oxidative state of tumour cells, which in turn influences the expression of regulatory microRNAs (miRNAs) (Mustafov et al., 2024).

1.12 MicroRNAs

MicroRNAs are small, naturally occurring, non-coding RNA molecules, usually around 18–22 nucleotides in length. They play an important role in regulating gene expression by either degrading messenger RNA (mRNA) or preventing it from being translated into proteins (Figure 5). Specifically, MicroRNAs (miRNAs) are small non-coding RNA molecules that function as important post-transcriptional regulators of gene expression. They are typically 18–22 nucleotides in length and regulate gene expression by binding to complementary messenger RNA (mRNA) sequences, resulting in either translational repression or degradation of the target mRNA (Enguita et al., 2022). Through these regulatory mechanisms, miRNAs are involved in essential biological processes including cell proliferation, differentiation, metabolism, apoptosis, and cellular stress responses (Hayes et al., 2014). Dysregulation of miRNA expression has been strongly associated with numerous diseases, particularly cancer, where altered miRNA activity contributes to tumour initiation, progression, invasion, and resistance to therapy (Hayes et al., 2014). MiRNA biogenesis begins in the nucleus, where miRNA genes are transcribed by RNA polymerase II into primary miRNA transcripts (pri-miRNAs) (Enguita et al., 2022). These pri-miRNAs are processed by the Drosha-DGCR8 microprocessor complex into precursor miRNAs (pre-miRNAs), which contain characteristic stem-loop structures. The pre-miRNAs are subsequently exported from the nucleus into the cytoplasm via Exportin-5. In the cytoplasm, the RNase III enzyme Dicer, together with TRBP, cleaves the pre-miRNA into a mature miRNA duplex. One strand of this duplex is then incorporated into the RNA-induced silencing complex (RISC), containing Argonaute proteins that guide the mature miRNA to its target

mRNA. Binding of the miRNA-RISC complex to target mRNA results in translational repression or mRNA degradation, thereby reducing protein expression (Enguita et al., 2022). The regulation of gene expression by miRNAs plays a critical role in maintaining normal cellular homeostasis. However, abnormal miRNA expression can occur through mechanisms such as mutations, chromosomal deletions, DNA methylation, or gene amplification, leading to disruption of normal cellular regulatory pathways (Hayes et al., 2014). In cancer biology, miRNAs are broadly classified into oncogenic miRNAs (oncomiRs) and tumour suppressor miRNAs. OncomiRs are typically overexpressed in cancer cells and promote tumour progression by suppressing tumour suppressor genes or apoptosis-related pathways. In contrast, tumour suppressor miRNAs are frequently downregulated in cancer, resulting in increased oncogene expression and enhanced tumour proliferation, invasion, and survival (Peng et al., 2016). Due to their ability to regulate multiple target genes simultaneously, miRNAs are recognised as major regulators of complex oncogenic signalling networks and have emerged as important biomarkers and therapeutic targets in cancer research (Karsy et al., 2012).

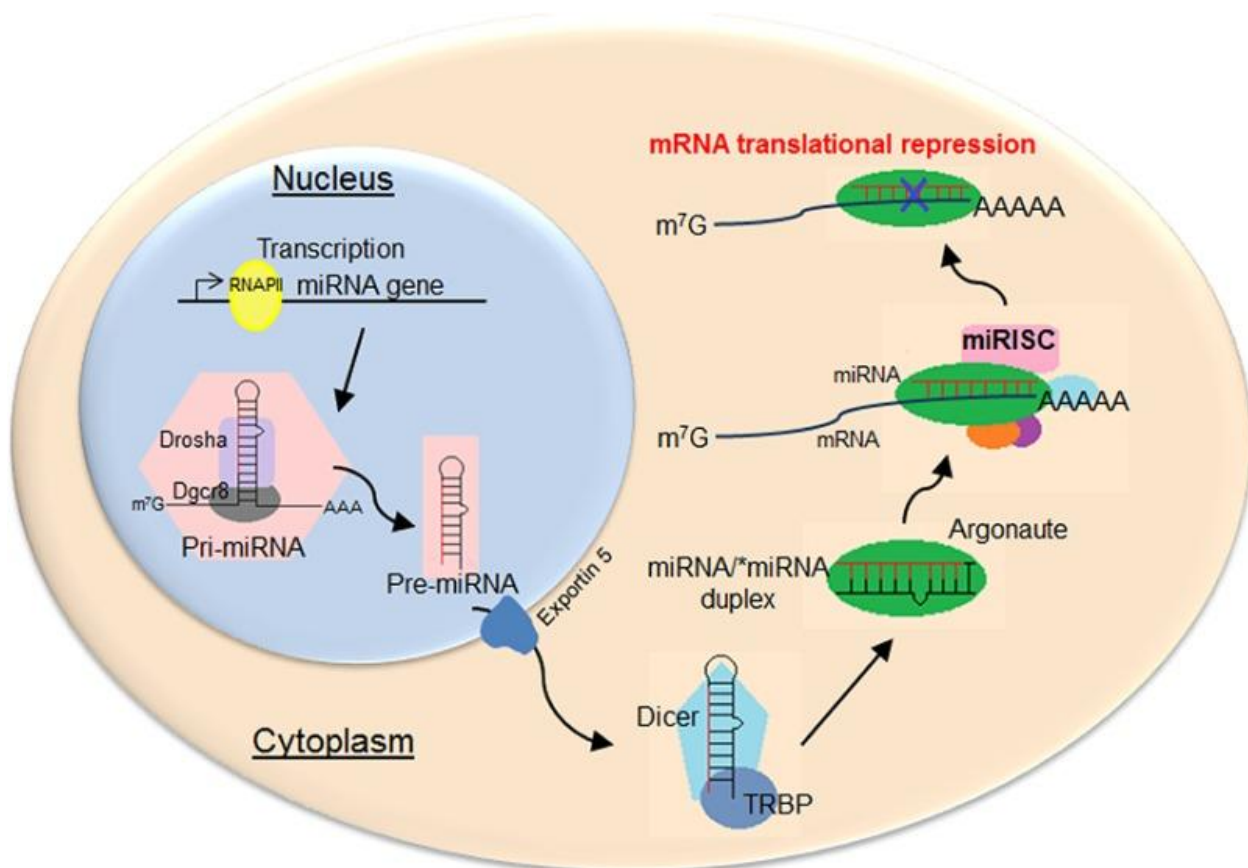


Figure 5: miRNA biogenesis and function. miRNAs are transcribed in the nucleus and processed into precursor forms before being exported to the cytoplasm, where they are

cleaved into mature miRNA. The mature miRNA is incorporated into the RISC complex and binds to target mRNA, leading to translational repression or degradation (Hajarnis et al., 2015).

Previously, the Braoudaki lab has shown that One key target of SHG-8 was miR-21, an oncogenic miRNA that is commonly overexpressed in glioblastoma. miR-21 promotes tumour progression by suppressing tumour suppressor genes, thereby increasing cell proliferation, invasion, and resistance to apoptosis. SHG-8 has been associated with the downregulation of miR-21, reducing its oncogenic activity. This modulation of miRNA expression also affects downstream targets such as CORO1C (Coronin-1C), an actin-binding protein involved in the Wnt/ β -catenin signaling pathway. CORO1C functions as an oncogene and contributes to glioblastoma progression by promoting cytoskeletal reorganization, cell motility, and invasion. By influencing miRNA expression, SHG-8 indirectly reduces CORO1C levels, thereby suppressing key pathways involved in tumour growth and spread. Alterations in miRNA expression play a central role in glioblastoma development (Mustafov et al., 2024).

1.13 3D Printing Technology and Its Applications

Personalized medicine, also known as precision medicine, is an emerging therapeutic strategy that aims to tailor treatments according to the specific biological and clinical characteristics of individual patients. This approach considers interpatient differences in pharmacokinetics and pharmacodynamics, which may result from variations in genetics, age, disease condition, and overall health status (Wang et al., 2023). In oncology, where patient responses to chemotherapy can vary significantly, precision medicine offers the potential to improve therapeutic efficacy while minimizing systemic toxicity and adverse side effects. Humanity finally realised that giving every patient the exact same treatment and hoping for the best was perhaps not the peak of scientific sophistication. Three-dimensional printing (3DP) has emerged as a highly promising technology within personalized medicine due to its ability to fabricate customized drug delivery systems with precise control over dose, geometry, drug distribution, and release kinetics (Awad et al., 2018a). As an additive manufacturing technique, 3DP constructs structures layer-by-layer using digitally guided computer-aided designs, enabling accurate control of material composition, microarchitecture, and surface properties during fabrication (Awad et al., 2018). This level of precision allows the development of dosage forms with tailored therapeutic characteristics that can be adapted to individual patient requirements. In pharmaceutical applications, 3DP enables the microengineering of drug release profiles and accurate drug loading by depositing active pharmaceutical ingredients directly within the internal structure of formulations, thereby protecting sensitive compounds from degradation and improving formulation stability (Vaz et al., 2021). Furthermore, 3DP technology supports the production of patient-specific medicines for paediatric, geriatric, and cancer populations through individualized dosing regimens and customizable dosage forms (Wang et al., 2023). The technology also facilitates rapid and on-demand manufacturing in clinical environments, reducing material waste and allowing immediate adaptation to patient-specific therapeutic needs (Awad et al., 2018). These advantages position 3DP as an important innovation in digital healthcare with the potential to improve therapeutic outcomes, enhance patient adherence, and transform the future of personalized cancer treatment.

1.14 Aims and Objectives

The aim of this study was to evaluate and elucidate the therapeutic effect of an in-house synthesized β -amino carbonyl (β -AC) compound, SHG-8, delivered in a 3D printed patch form, using U87MG glioblastoma cell line models. To achieve this, a series of experimental and analytical approaches were undertaken. The cytotoxic effects of SHG-8 were assessed using the MTT assay to determine its impact on cell viability across different concentrations. The effect of the compound on cell migration was evaluated using the scratch assay, while its influence on long-term cell survival and proliferative capacity was investigated through the colony formation assay. In addition, small RNA (sRNA) sequencing was performed to identify changes in miRNA expression profiles following SHG-8 treatment. The data were further analyzed using bioinformatic tools to predict target genes and identify key signaling pathways associated with glioblastoma progression. Together, these approaches aimed to provide a comprehensive understanding of the potential mechanism of action of SHG-8, particularly through miRNA-mediated regulatory pathways.

2. Methods and Materials

2.1 Synthesis of SHG-8

The SHG-8 drug was synthesized by our collaborators at the American University of Ras Al Khaimah (Ras Al Khaimah, UAE) following established synthetic protocols. The target compound SHG-8 was produced via a one-pot Mannich reaction catalyzed by 0.04 g of SAFSNS. The reaction involved acetophenone (1.1 mmol), 4-bromobenzaldehyde (1 mmol), and aniline (1 mmol) in ethanol at room temperature, stirred continuously for four hours. Reaction progress was monitored using thin-layer chromatography (TLC). Upon completion, the solvent was evaporated, yielding a yellow solid. The catalyst was recovered by filtration after dissolving the crude product in dichloromethane at 35 °C. Finally, the product was purified by recrystallization from ethanol, resulting in a light-yellow solid. The preparation, purification, and characterization of the drug were conducted entirely at their facility prior to its use in this study.

2.2 Cell Culture

Glioblastoma U87MG cells, originating from malignant gliomas, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured using a complete medium of Gibco's Minimum Essential Media (MEM, Gibco, Bleiswijk, Netherlands) as per the manufacturer's instructions and supplemented with 1% penicillin-streptomycin (Gibco, Bleiswijk, Netherlands) and 10% Foetal Bovine Serum (Gibco, Bleiswijk, Netherlands) under sterile conditions. Cultures were incubated at 37 °C in an atmosphere containing 5% CO₂. All media changes and passaging were performed in a fume hood, using aseptic techniques. U87MG cells were routinely monitored for confluency and morphology using an electronic microscope and were checked for contamination before every experiment. Media changes were performed every 48-72 hours. Passaging was carried out at 70–80% confluency by washing with phosphate-buffered saline (DPBS; Thermo Fisher Scientific, Waltham, Massachusetts, USA) detaching cells using 0.25% trypsin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and neutralizing the cells with fresh medium.

2.3 Thermogravimetric analysis of SHG-8

Thermogravimetric analysis of b3-(4-bromophenyl)-1-phenyl-3-(phenylamino) propan-1-one (SHG-8), was performed using a TA instrument Q50 TGA – as shown below (Figure 6), under a nitrogen atmosphere to prevent oxidative degradation. Approximately 6mg of SHG-8 was weighed using analytical balance and placed in a clean platinum pan. The sample was heated from room temperature to 600 °C at a rate of 10 °C/min under a continuous nitrogen flow of 50 mL/min. The weight loss of the sample was continuously recorded to assess its thermal stability and decomposition pattern.

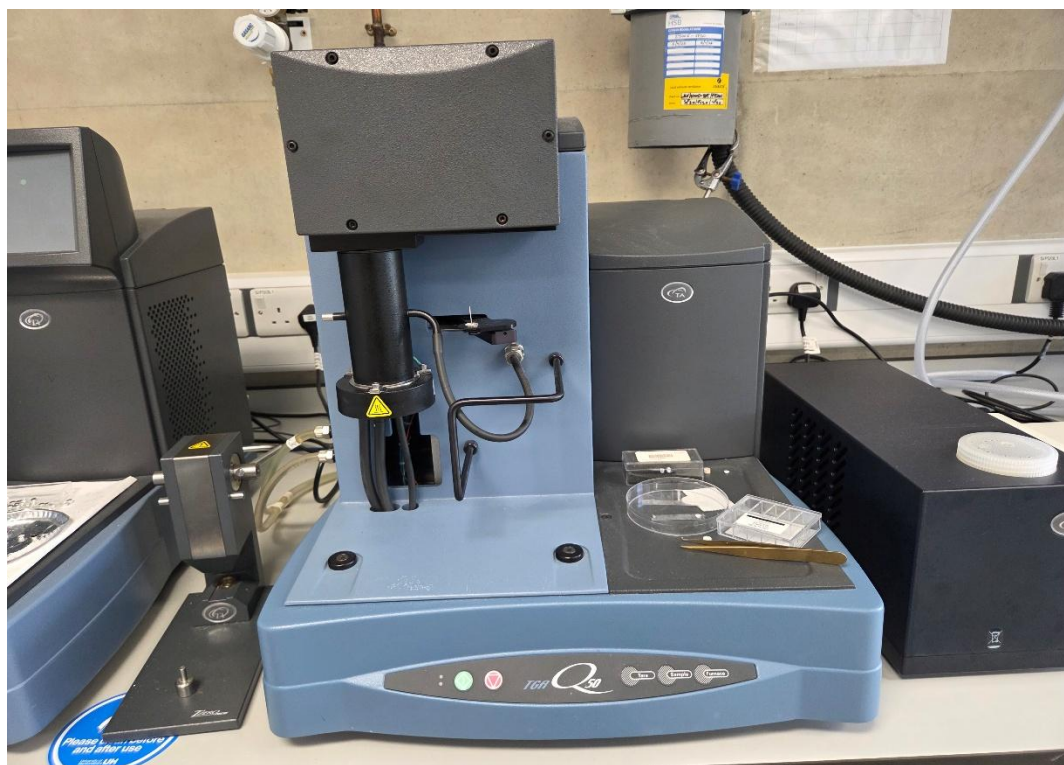


Figure 6: TA Instrument Q50 Thermogravimetric Analyzer (TGA) (TA Instrument, New Castle, Delaware, USA). TGA was used to measure the thermal degradation profile of the SHG-8 drug by monitoring weight loss as a function of temperature under a controlled nitrogen atmosphere, ensuring accurate characterization of its decomposition behavior.

2.4 Gel Preparation for 3D printing of SHG-8 drug patches

A drug-free gel was first prepared using 4% HPMC K100 M, 1% NaCMC 20,000PA, 1% glycerol, and 94% distilled water to standardize the 3D printing process. Nine patches were printed using this base formulation and individually weighed with a precision microbalance to calculate the average patch mass. This average weight was used to determine the required amount of SHG-8 drug needed per patch. Based on these calculations, three gel formulations were prepared for 3D printing of drug patches, each containing different concentrations of SHG-8 (25 μ M, 50 μ M, and 100 μ M), corresponding to 1% (0.1 g), 2% (0.2 g), and 3% (0.3 g) w/w respectively, in a total gel mass of 10 g. All formulations also included 4% (0.4 g) hydroxypropyl methylcellulose (HPMC K100 M), 1% (0.1 g) sodium carboxymethyl cellulose (NaCMC 20,000PA), 1% (0.1 g) glycerol, and the remaining amount of distilled water—9.3 g, 9.2 g, and 9.1g respectively. For each preparation, half of the required distilled water was weighed and heated to 80 °C for 3 minutes. The weighed amounts of HPMC and NaCMC were then gradually added to the warm water. The mixture was stirred using a magnetic stirrer at 500 rpm for 5 minutes to ensure full dissolution of the polymers. The remaining volume of distilled water was added and stirring continued for an additional 5 minutes. The mixture was then vortexed until a visibly homogeneous gel formed. Glycerol was subsequently added to the gel, followed by incubation at room temperature for 2 hours to allow for gel solidification. A separate gel-mix was prepared for each SHG-8 concentration.

2.5 3D printing of SHG-8 drug patches

Each gel formulation was loaded into a 3 mL cartridge, which was then placed onto the pneumatic 3 mL printhead (Figure 7) of the CELLINK BIO X 3D bioprinter (CELLINK, Gothenburg, Sweden) which is shown below (Figure 7A). A 22-gauge (0.410 mm) nozzle was attached for all prints. A computer-aided design (CAD) model of nine square patches, each measuring 8.5 mm (width) × 8.5 mm (length) with a height of 0.2 mm, was created using the 3D design software Tinkercad and uploaded to the printer via USB. Printing parameters were set as follows: temperature at 37 °C, pressure at 15 kPa, speed at 20 mm/s, layer height of 0.20 mm, and 100% infill using a grid pattern. Manual calibration was performed by positioning Tool 1 at the desired starting point on the print surface. For each printing session, a sterile 100 mm diameter Petri dish was used as the substrate (Figure 3B). Each Petri dish containing the nine printed patches was carefully enclosed in a plastic bag and then placed in a refrigerator at 4 °C for incubation over 24 hours.

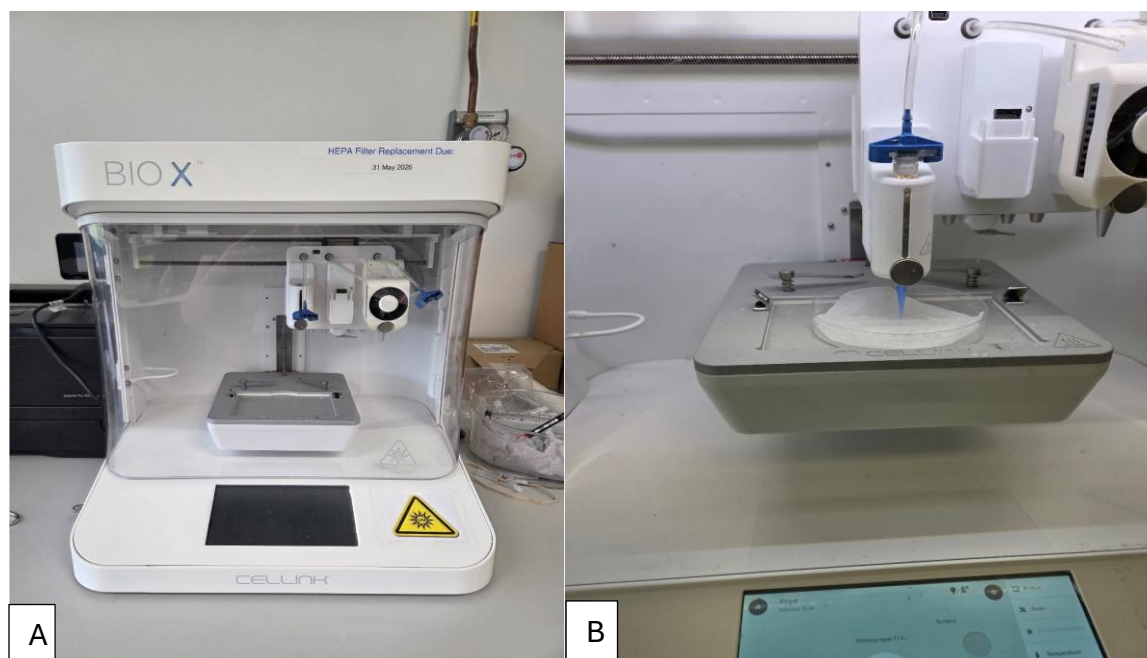


Figure 7: CELLINK BIO X 3D Bioprinter (CELLINK, Gothenburg, Sweden). **A:** The printer was used to make 3D SHG-8 drug patches with high accuracy, allowing precise placement of the bioink for targeted drug delivery. **B:** Pneumatic Printhead Setup with 3 mL Cartridge on the CELLINK Bio X Printer. The figure shows the Pneumatic Printhead, also referred as Tool 1, loaded with a 3 mL cartridge which is attached with an air adapter connector, and positioned above a petri dish on the printer base.

2.6 MTT cell viability assay

U87MG cells were seeded into 96-well plates at a density of 15,000 cells per well. Two separate plates were prepared: in the first plate, cells were treated with increasing concentrations of SHG-8 drug patches at 25 μ M, 50 μ M, and 100 μ M. In the second plate, cells were treated with SHG-8 drug patches prepared at the same concentration range. DMSO was used as a negative control, while cisplatin at 200 μ M served as a positive control. Following 24 hours of incubation at 37 °C and 5% CO₂, 5 mg/mL of thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, USA) reagent was added to each well, and the plates were further incubated for 2 hours. After incubation, the MTT solution was removed, and 100% isopropanol was added to each well to dissolve the formazan crystals. Plates were placed on a rocking platform for 30 minutes to ensure complete dissolution. Absorbance was measured at 570 nm using a CLARIOstar microplate reader (BMG LABTECH, Offenburg, Germany), as shown below, to assess cell viability.

2.8 Colony Formation Assay

U87MG cells were seeded in triplicate into 12-well plates at a density of 500 cells per well and were treated with SHG-8 drug patches at concentrations of 40 μ M and 60 μ M, while cells treated with <1% DMSO and 200 μ M cisplatin served as a negative control and positive control, respectively, and were incubated in complete growth media for 24 h. Following treatment exposure, the drug-supplemented media was removed, and the cells were further incubated for five days at 37°C in a humidified atmosphere containing 5% CO₂ to allow colony formation. At the end of the incubation period, colonies were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich TM, Dorset, UK) for 25 min and subsequently stained using 0.1% crystal violet (Sigma-Aldrich™, Dorset, UK). Stained colonies were washed with distilled water and left to air dry overnight. Colonies consisting of more than 30 cells were microscopically photographed and quantitatively assessed using a light microscope (Olympus Life Science Solutions, Stansted, UK) to evaluate the effects of treatment on the clonogenic survival and proliferative capacity of U87MG cells.

2.9 small RNA sequencing

Small RNA sequencing was performed to identify differentially expressed microRNAs (miRNAs) associated with GB. Total RNA, including the small RNA fraction, was isolated from experimental samples using standard RNA extraction protocols under RNase-free conditions. RNA quantity and integrity were assessed prior to library preparation to ensure sample quality for downstream sequencing analysis. RNA samples were subsequently sent to Biomarker Technologies (BMKGene; <https://www.bmkgene.com/>) for small RNA library preparation and sequencing analysis according to the company's standard protocols. Following sequencing, raw sequencing reads were subjected to quality control assessment, adaptor trimming, and filtering to remove low-quality reads and contaminants prior to downstream analysis. Processed sequencing data were normalized and analyzed to identify differentially expressed miRNAs associated with glioblastoma pathogenesis and progression.

1.10 Bioinformatics analysis

Bioinformatic analysis was conducted to identify miRNAs with potential clinical relevance for the diagnosis, prognosis, and therapeutic targeting of glioblastoma (GBM). Differentially expressed miRNAs identified through small RNA sequencing analysis were further analyzed using the miRSystem database (<http://mirsystem.cgm.ntu.edu.tw/index.php>) to generate a report of target genes. Only downregulated miRNAs identified from the sequencing dataset were input into the database for target prediction analysis. To improve the reliability of the predicted interactions, the default miRSystem parameter was configured to require a minimum of three algorithm hits. Target genes were analyzed for their involvement in glioblastoma and their relevance in tumorigenesis using the Kyoto Encyclopedia of Genes and Genomes (KEGG; <https://www.genome.jp/kegg/pathway.html>). Functional pathway enrichment analysis was performed to investigate the association of differentially expressed miRNAs and their target genes with glioblastoma development and tumorigenesis.

2.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The MTT cell viability assay, scratch assay, and colony formation assay were performed using SHG-8 3D-printed drug patches. Each treatment condition was assessed using five technical replicates within triple biological replicates. Quantitative data obtained from the assays were expressed as mean $\pm$ standard deviation (SD). Statistical differences between treatment groups were evaluated using one-way analysis of variance (ANOVA) and post hoc Tukey's test on SPSS. Statistical significance was defined as a p-value of less than 0.05.

3. Results

3.1 U87MG Glioblastoma cells

In Figure 8, a representation of U87MG glioblastoma cells is shown that were cultured under standard conditions, reaching approximately 50%-70% confluency. Cells exhibited an adherent growth pattern irregular, polygonal cell bodies and cytoplasmic projections. Intercellular contacts are visible but incomplete, and the monolayer remains discontinuous, characteristic of a proliferating, sub-confluent glioblastoma culture.

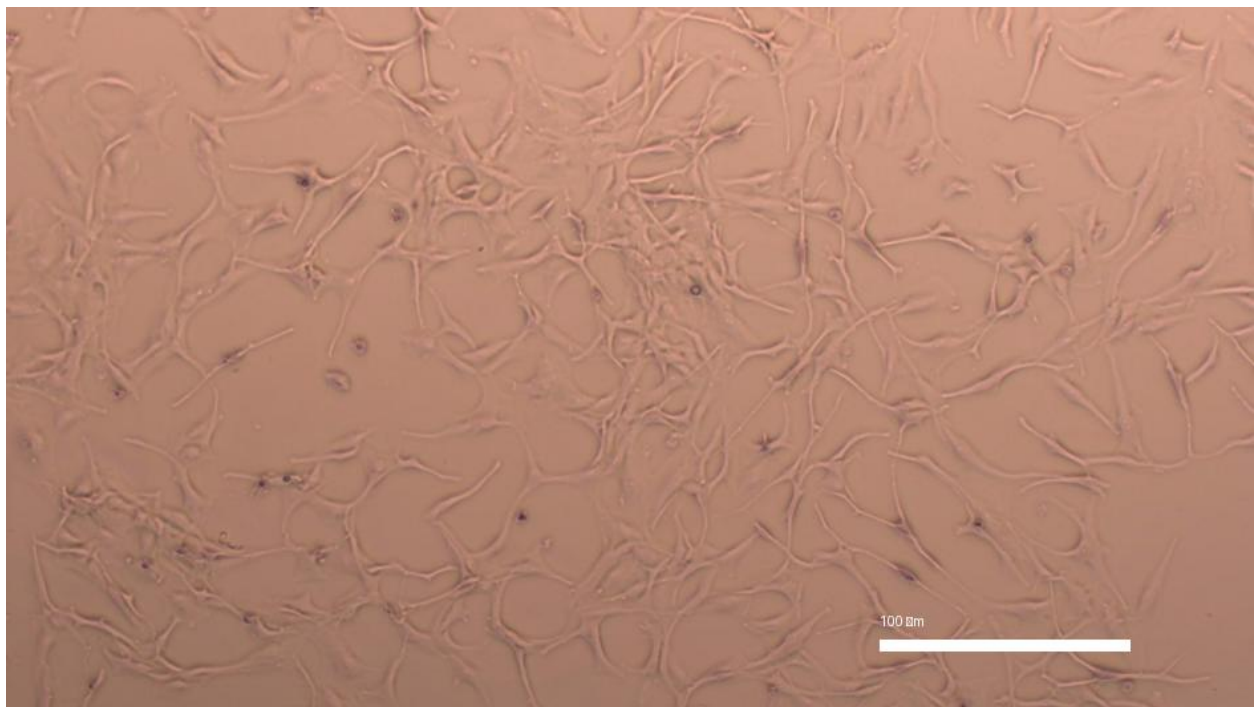


Figure 8: Light microscopy images showing the morphology of U87MG glioblastoma cells after 48 hours of culture. A total magnification of 40x was used to observe U87MG cells.

3.2 Thermogravimetric Analysis of SHG-8 drug

Figure 9 presents the TGA curve of the sample SGH-8 drug, with an initial mass of 2.1400 mg. The x-axis represents the time (min) and corresponding temperature (°C), while the y-axis shows the normalized weight (%) of the sample. The TGA curve indicated that the sample remained thermally stable across the measured temperature range (up to 190 °C), with negligible weight loss. A slight downward trend can be observed at the upper end of the temperature scale, which corresponds to the onset of degradation, suggesting that significant decomposition would occur if the sample were heated beyond 190 °C.

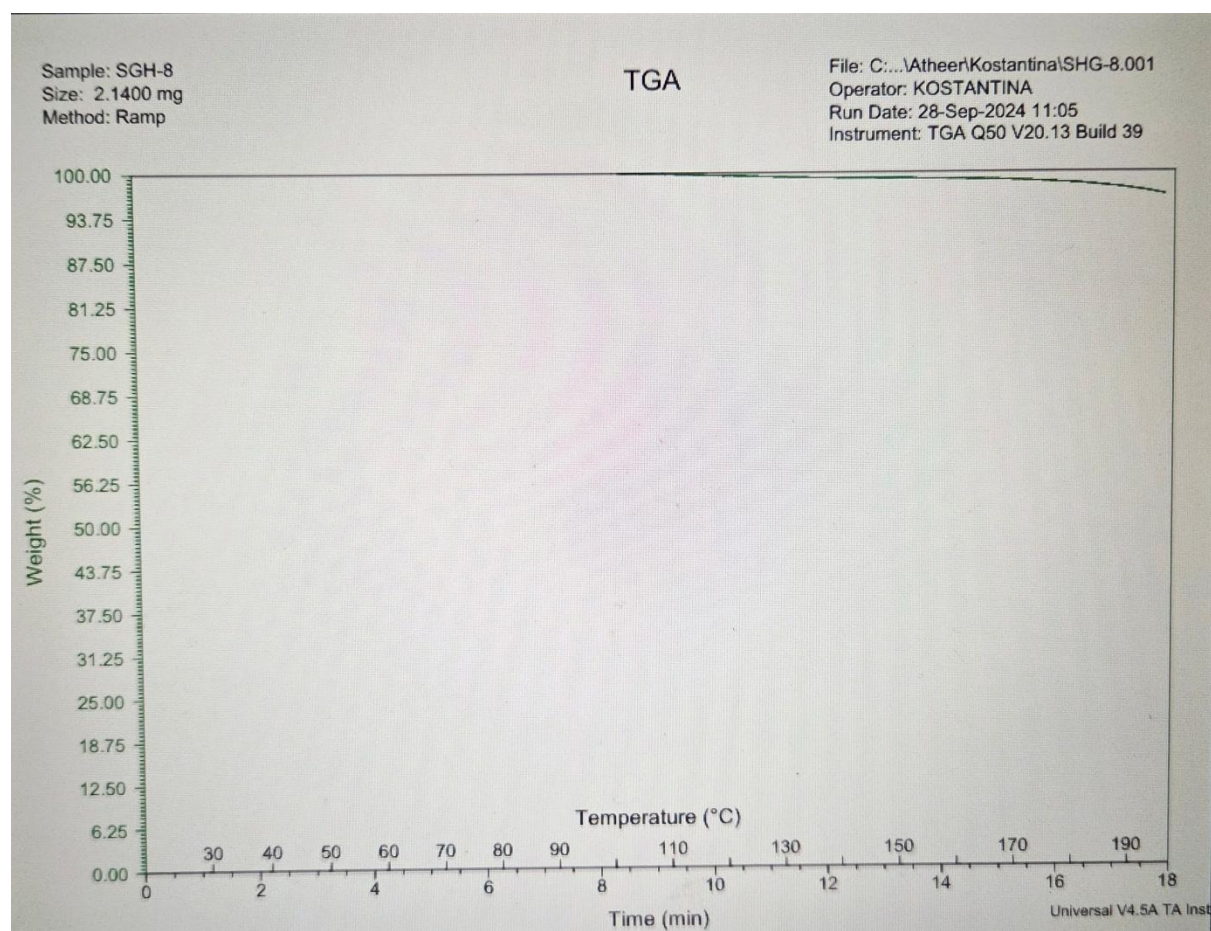


Figure 9: Thermogravimetric Analysis (TGA) of SGH-8 drug. Degradation point of SHG-8 drug at 190 °C.

3.3 3D Printing of SHG-8 drug patches

Figure 10 shows the 3D printed SHG-8 drug patches were uniformly printed, with consistent size, shape, and thickness. The hydrogel-based patches, composed of 4% HPMC, NaCMC, water, and glycerol, exhibited smooth surfaces and clear edges. This setup allowed for parallel testing of multiple patches under controlled conditions, providing a reliable platform to assess drug release and cytotoxic effects in U87MG glioblastoma cell cultures.

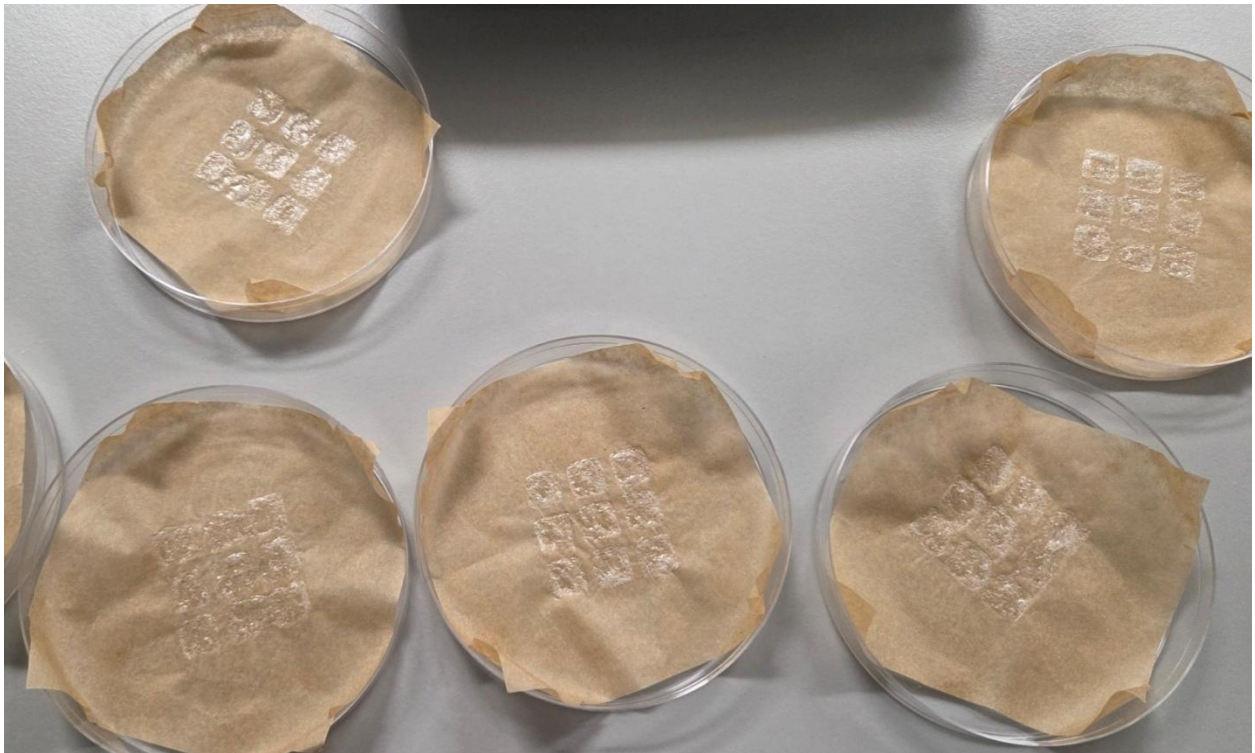


Figure 10: Representation of five petri dishes containing SHG-8 3D printed patches. Each petri dish containing nine 3D printed SHG-8 drug patches.

3.4 MTT cytotoxic assay

Quantitative analysis demonstrated differences in cell viability between the control and treatment groups, with reduced average viability values observed across the SHG-8 patch-treated conditions relative to the DMSO control group. Statistical analysis using one-way ANOVA followed by multiple comparison testing identified significant differences between selected treatment groups. The viability data generated were subsequently used for graphical representation and IC50 estimation analyses, as presented in Table 1, Figure 9, and Table 2.

Table 1 presents the cell viability percentages obtained from the MTT assay performed in U87MG cells following treatment with SHG-8 patch and control. Average cell viability values of 100%, 61%, 49%, 49%, and 42% were recorded for the DMSO control, 200 μ M cisplatin, 25 μ M SHG-8 patch, 50 μ M SHG-8 patch, and 100 μ M SHG-8 patch treatment groups, respectively. Standard deviation percentages were calculated across biological replicates and were recorded as 0.00%, 17.56%, 11.59%, 3.27%, and 24.75% for each corresponding treatment condition. The table summarises the viability values obtained for each biological replicate together with the calculated average and standard deviation values across all treatment groups.

Table 1. Cell Viability (%) of U87MG Cells Following Treatment with SHG-8 Patch and Control Conditions					
Parameter	DMSO	Cisplatin (200 μ M)	SHG-8 Patch (25 μ M)	SHG-8 Patch (50 μ M)	SHG-8 Patch (100 μ M)
N1 % Viability	100%	39%	62%	45%	7%
N2 % Viability	100%	82%	34%	51%	59%
N3 % Viability	100%	61%	52%	49%	60%
Average Cell Viability (%)	100%	61%	49%	49%	42%
Standard Deviation (%)	0.00%	17.56%	11.59%	3.27%	24.75%

The data presented in Table 1 were used to generate the graphical representation shown in Figure 9, illustrating the effect of SHG-8 patch treatment on the viability of U87MG cells following MTT assay analysis. Average cell viability percentages obtained from three independent biological replicates were plotted for the DMSO control, 200 μ M cisplatin, and SHG-8 patch treatment groups at concentrations of 25 μ M, 50 μ M, and 100 μ M. The DMSO control group demonstrated the highest average viability at 100%. Treatment with 200 μ M cisplatin resulted in an average viability of 61%. Treatment with SHG-8 patch at 25 μ M produced an average cell viability of 49%, while treatment at 50 μ M also resulted in an average viability of 49%. The 100 μ M SHG-8 patch-treated group exhibited the lowest average cell viability at 42%. Standard deviation values associated with each treatment group were included as error bars within the graph. Statistical analysis was performed using one-way ANOVA followed by multiple comparison testing to evaluate differences between treatment groups relative to the DMSO control condition. No significant difference was observed between the DMSO control group and the 200 μ M cisplatin-treated group (ns). Significant differences were identified between the DMSO control group and the 25 μ M SHG-8 patch-treated group (* p < 0.05), between the DMSO control group and the 50 μ M SHG-8 patch-treated group (* p < 0.05), and between the DMSO control group and the 100 μ M SHG-8 patch-treated group (* p < 0.05). Comparisons between the SHG-8 patch treatment groups demonstrated average viability values of 49%, 49%, and 42% for the 25 μ M, 50 μ M, and 100 μ M treatment conditions, respectively, as presented in Figure 9.

Effect of SHG-8 patch treatment on U87MG cell viability

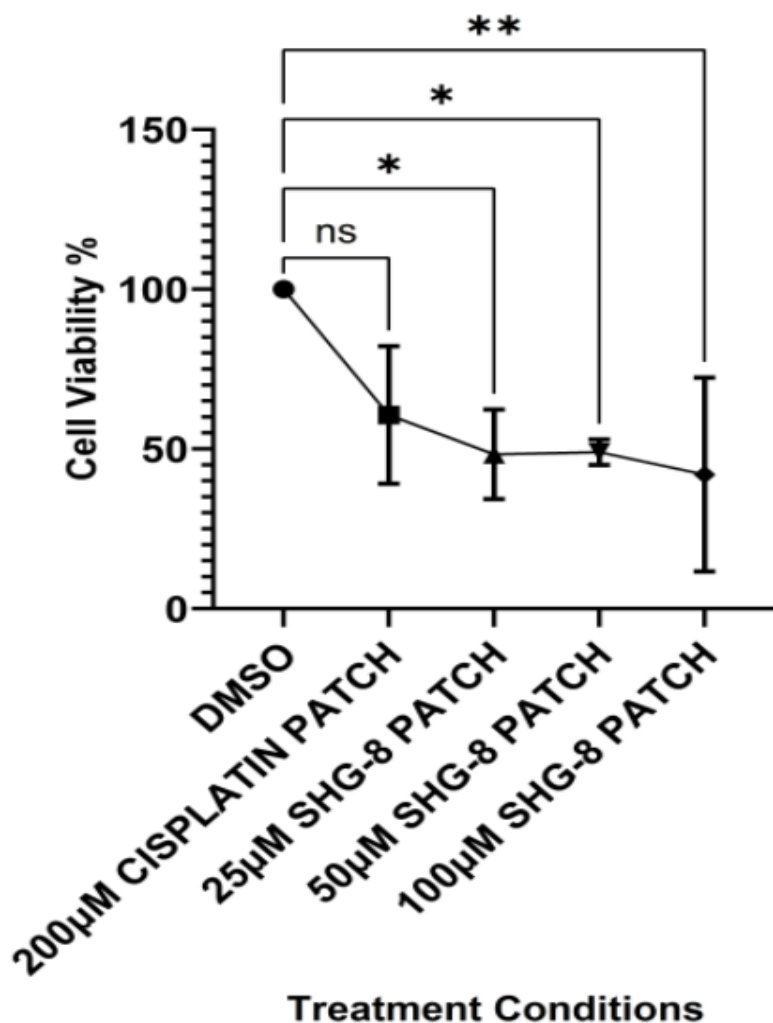


Figure 9: Effect of SHG-8 Patch Treatment on U87MG Cell Viability: Figure 9 presents the percentage cell viability of U87MG cells following treatment with DMSO control, 200 µM cisplatin, and SHG-8 patch at concentrations of 25 µM, 50 µM, and 100 µM, as determined using three independent biological replicates, each containing five technical replicates per treatment condition. The DMSO control group showed a cell viability value of 100%. Treatment with 200 µM cisplatin resulted in a mean cell viability of approximately 61%. Treatment with SHG-8 patch produced mean cell viability values of approximately 49% at 25 µM, 49% at 50 µM, and 42% at 100 µM. Error bars represent standard deviation values calculated across biological replicates. Statistical analysis was performed using one-way ANOVA followed by multiple comparison analysis. No significant difference was observed between the DMSO control group and the 200 µM cisplatin-treated group (ns). Significant differences were identified between the DMSO control group and the 25 µM SHG-8 patch-

treated group (*p < 0.05), between the DMSO control group and the 50 μ M SHG-8 patch-treated group (*p < 0.05), and between the DMSO control group and the 100 μ M SHG-8 patch-treated group (**p < 0.01).

Following the analysis of cell viability across the different SHG-8 patch treatment concentrations, dose-response data obtained from the MTT assay were subsequently used to estimate the IC₅₀ values for each independent biological replicate. IC₅₀ estimation was performed using the viability percentages generated from the treatment groups to determine the concentration of SHG-8 associated with a 50% reduction in cell viability relative to the control condition. The calculated IC₅₀ values obtained from the three biological replicates are presented in Table 2. Specifically, Table 2 presents the estimated IC₅₀ values obtained from three independent biological replicates (N1, N2, and N3) of the MTT assay performed in U87MG cells following treatment with SHG-8. Estimated IC₅₀ values of 50.0 μ M, 62.7 μ M, and 41.7 μ M were obtained for biological replicates N1, N2, and N3, respectively. The calculated mean IC₅₀ value across all biological replicates was 51.47 μ M.

Table 2. Estimated IC₅₀ Values of SHG-8 in U87MG Cells

Biological Replicate	Estimated IC ₅₀ (μ M)
N1	50.0
N2	62.7
N3	41.7
Mean $\pm$ SD	51.47 $\pm$ 10.5

3.5 Colony formation

Colony formation assays were performed to assess the effect of SHG-8-loaded patches on the long-term clonogenic growth and survival of U87MG glioblastoma cells. U87MG cells were treated with DMSO control, 200 μ M cisplatin-loaded patches, 40 μ M SHG-8-loaded patches, and 60 μ M SHG-8-loaded patches, and colony formation was quantified following the incubation period. The assay was conducted across three independent biological replicates (N1–N3), with each biological replicate consisting of three technical replicate wells per treatment condition. Colony counts obtained from individual wells were averaged within each biological replicate prior to statistical analysis. Across the three biological replicates, the DMSO control group demonstrated the highest colony counts, with an overall average colony count of 58.2 colonies. In comparison, reduced colony counts were observed following treatment with 200 μ M cisplatin-loaded patches, 40 μ M SHG-8-loaded patches, and 60 μ M SHG-8-loaded patches, with overall average colony counts of 20.54, 7.00, and 0.66 colonies, respectively. The lowest colony counts were consistently observed in the 60 μ M SHG-8 patch-treated group across all biological replicates. Quantitative analysis further demonstrated progressive reductions in colony numbers between the DMSO control group, cisplatin-treated group, and SHG-8-treated groups. Statistical analysis demonstrated a significant difference among treatment group means ($p = 0.0224$). Significant reductions in colony counts were identified in both the 40 μ M SHG-8 patch-treated group and the 60 μ M SHG-8 patch-treated group relative to the DMSO control group ($*p < 0.05$). In contrast, no statistically significant difference was identified between the DMSO control group and the 200 μ M cisplatin-treated group (ns). Individual colony counts obtained from each biological replicate, together with averaged treatment values and graphical statistical comparisons, are presented in Tables 2–5 and Figure 13.

Table 2 presents the colony counts obtained from the first biological replicate (N1) of the colony formation assay performed in U87MG cells following treatment with DMSO control, 200 μ M cisplatin, 40 μ M SHG-8 patch, and 60 μ M SHG-8 patch conditions.

Table 2. Colony Formation Counts Following Treatment in Biological Replicate N1					
No.	Well	DMSO	200 μM Cisplatin	40 μM SHG-8 Patch	60 μM SHG-8 Patch
1.	Well 1	120	54	15	0
2.	Well 2	95	22	6	0
3.	Well 3	68	33	18	1
4.	Average	94.3	36.3	13.0	0.33

Table 3 presents the colony counts obtained from the second biological replicate (N2) of the colony formation assay performed in U87MG cells. Colony counts were measured across DMSO control, 200 μ M cisplatin, 40 μ M SHG-8 patch, and 60 μ M SHG-8 patch treatment groups. Average colony counts of 26.3, 7.33, 3.66, and 1.0 were recorded for the respective treatment conditions.

Table 3. Colony Formation Counts Following Treatment in Biological Replicate N2					
No.	Well	DMSO	200 μM Cisplatin	40 μM SHG-8 Patch	60 μM SHG-8 Patch
1.	Well 1	24	8	3	2
2.	Well 2	32	1	4	0
3.	Well 3	23	13	4	1
4.	Average	26.3	7.33	3.66	1.0

Table 4 presents the colony counts obtained from the third biological replicate (N3) of the colony formation assay performed in U87MG cells following treatment with DMSO control, 200 μ M cisplatin, 40 μ M SHG-8 patch, and 60 μ M SHG-8 patch conditions.

Table 4. Colony Formation Counts Following Treatment in Biological Replicate N3					
No.	Well	DMSO	200 μM Cisplatin	40 μM SHG-8 Patch	60 μM SHG-8 Patch
1.	Well 1	51	11	7	0
2.	Well 2	43	7	4	2
3.	Well 3	68	36	2	0
4.	Average	54.0	18.0	4.33	0.66

Table 5 summarizes the average colony counts obtained from three independent biological replicates (N1–N3) of the colony formation assay performed in U87MG cells. Each reported value represents the mean colony count derived from three technical replicate wells within each biological replicate. Colony counts were recorded following treatment with DMSO control, 200 μ M cisplatin-loaded patches, 40 μ M SHG-8-loaded patches, and 60 μ M SHG-8-loaded patches. The table demonstrates the quantified average colony counts across all treatment conditions and biological replicates used for subsequent statistical analysis and graphical presentation.

Table 5. Average Colony Formation Counts Across Biological Replicates (N1–N3)				
No.	Treatment Condition	Biological Replicate N1 (Average)	Biological Replicate N2 (Average)	Biological Replicate N3 (Average)
1.	DMSO	94.3	26.3	54.0
2.	200 μM Cisplatin	36.3	7.33	18.0
3.	40 μM SHG-8 Patch	13.0	3.66	4.33
4.	60 μM SHG-8 Patch	0.33	1.0	0.66

Colony formation analysis was performed to assess the clonogenic growth of U87MG cells following treatment with 200 μ M cisplatin-loaded patches, 40 μ M SHG-8-loaded patches, and 60 μ M SHG-8-loaded patches, in comparison with the DMSO control group. Quantitative colony counts obtained from three independent biological replicates were analyzed and compared between treatment groups. Average colony counts of 58.2, 20.54, 7.00, and 0.66 were observed for the DMSO, 200 μ M cisplatin, 40 μ M SHG-8 patch, and 60 μ M SHG-8 patch treatment groups, respectively. Statistical analysis demonstrated a significant difference among treatment group means ($p = 0.0224$). Significant reductions in average colony count were observed in both the 40 μ M SHG-8 patch-treated group and the 60 μ M SHG-8 patch-treated group relative to the DMSO control group ($*p < 0.05$), whereas no significant difference was identified between the DMSO control and the 200 μ M cisplatin-treated group (ns). The quantified colony counts and corresponding statistical comparisons are presented in Figure 13.

Effect of SHG-8 patch treatment on colony formation in U87MG cells

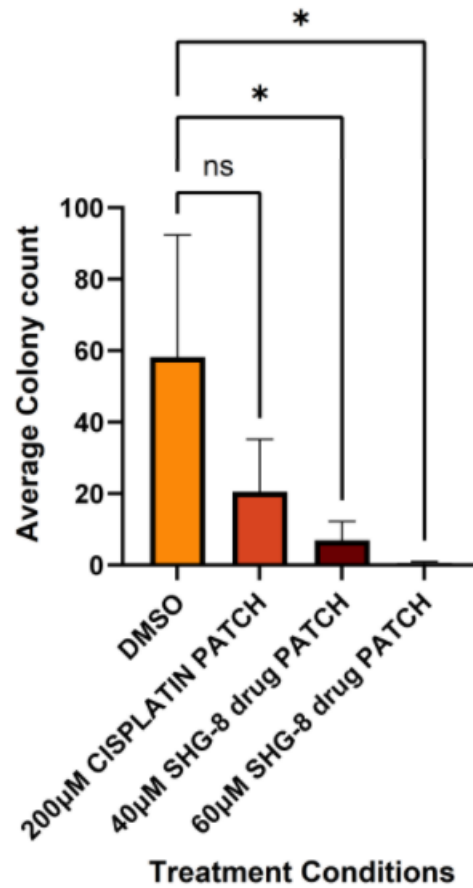


Figure 13: Effect of SHG-8 patch treatment on colony formation in U87MG cells. Colony formation assays were performed to evaluate the effect of SHG-8-loaded patches on the clonogenic capacity of U87MG cells. Quantitative analysis demonstrated average colony counts of 58.2 for the DMSO control group, 20.54 for the 200 µM cisplatin-treated group, 7.00 for the 40 µM SHG-8 patch-treated group, and 0.66 for the 60 µM SHG-8 patch-treated group. One-way ANOVA analysis demonstrated a statistically significant difference among treatment group means ($p = 0.0224$). Comparison of treatment groups relative to the DMSO control demonstrated no significant difference between DMSO and 200 µM cisplatin treatment (ns), while significant differences were identified between DMSO and the 40 µM SHG-8 patch treatment ($*p < 0.05$), and between DMSO and the 60 µM SHG-8 patch treatment ($*p < 0.05$). Additionally, colony counts observed following 40 µM SHG-8 patch treatment were greater than those observed following 60 µM SHG-8 patch treatment. Data are presented as mean $\pm$ standard deviation (SD) from three independent biological replicates.

3.6 Small RNA sequencing

Small RNA sequencing analysis was performed to identify differentially expressed miRNAs in U87MG cells following SHG-8 drug patch treatment. Sequencing data identified multiple miRNAs exhibiting altered expression profiles relative to the control condition. Differential expression analysis demonstrated a subset of miRNAs that were significantly downregulated and were subsequently selected for further bioinformatic investigation. The sequencing analysis generated expression profiles for miRNAs associated with cellular regulatory processes involved in glioblastoma biology. Identified downregulated miRNAs were compiled and ranked according to differential expression patterns for subsequent target gene prediction and pathway enrichment analysis. The resulting data provided a profile of altered miRNA expression within treated U87MG cells.

Table 5 presents the list of miRNAs identified as downregulated in U87MG cells following treatment with the SHG-8 drug, as determined by small RNA sequencing analysis. A total of 38 miRNAs showed reduced expression compared to the control condition. These miRNAs are involved in post-transcriptional regulation of gene expression, and their downregulation suggests that SHG-8 treatment may alter multiple regulatory pathways associated with cell proliferation, survival, and tumour progression.

Table 5: Downregulated miRNAs following SHG-8 treatment.

No.	Downregulated miRNAs Following SHG-8 Treatment
1.	hsa-miR-125b-1-3p
2.	hsa-miR-23a-5p
3.	hsa-miR-27a-5p
4.	hsa-miR-27b-5p
5.	hsa-miR-1303
6.	hsa-miR-92a-1-5p
7.	hsa-miR-25-5p
8.	hsa-miR-92b-5p
9.	hsa-miR-1268a
10.	hsa-miR-138-1-3p
11.	hsa-miR-671-3p
12.	hsa-miR-191-3p
13.	hsa-miR-30d-3p
14.	hsa-miR-365b-5p
15.	hsa-miR-222-3p
16.	hsa-miR-184
17.	hsa-miR-32-3p
18.	hsa-miR-551b-5p
19.	hsa-miR-941
20.	hsa-miR-221-5p
21.	hsa-miR-130b-5p
22.	hsa-miR-494-5p
23.	hsa-miR-19b-1-5p
24.	hsa-miR-10a-5p
25.	hsa-miR-4435
26.	hsa-miR-128-3p
27.	hsa-miR-484
28.	hsa-miR-421
29.	hsa-miR-3124-5p
30.	hsa-miR-1249-3p
31.	hsa-miR-1260b
32.	hsa-miR-92b-3p
33.	hsa-miR-9-5p
34.	hsa-miR-20a-5p
35.	hsa-miR-10b-5p
36.	hsa-miR-942-5p
37.	hsa-miR-361-3p
38.	hsa-miR-155-3p

3.7 Bioinformatic analysis

Bioinformatic analysis identified multiple downregulated miRNAs following treatment, as determined by sRNA sequencing analysis. Differentially expressed miRNAs were subsequently analyzed to identify predicted target genes and associated signaling pathways. The analysis identified target genes involving several glioblastoma-related pathways, including PI3K/AKT, MAPK, Wnt/ β -catenin, FOXO, Hippo, and TGF- β signaling pathways. Multiple tumour suppressor genes and regulatory genes, including *PTEN*, *FOXO4*, *YAP1*, *LATS1*, and *TRIM33*, were identified among the predicted targets. Overlapping target genes were additionally identified between selected miRNAs through comparative analysis, and common target genes were visualized using Venn diagrams. KEGG pathway maps further highlighted the involvement of selected target genes within glioma-associated molecular signaling pathways.

Table 6 presents the downregulated miRNAs identified following SHG-8 treatment in U87MG cells that did not demonstrate predicted target genes following bioinformatic analysis using the miRSystem database. Although these miRNAs were significantly downregulated according to the small RNA sequencing data, no associated target genes were identified.

Table 6: miRNAs with no Target Genes following SHG-8 treatment.

No.	miRNAs with No Predicted Target Genes Following SHG-8 Treatment
1.	hsa-miR-125b-1-3p
2.	hsa-miR-23a-5p
3.	hsa-miR-27a-5p
4.	hsa-miR-27b-5p
5.	hsa-miR-1303
6.	hsa-miR-92a-1-5p
7.	hsa-miR-25-5p
8.	hsa-miR-1268a
9.	hsa-miR-138-1-3p
10.	hsa-miR-671-3p
11.	hsa-miR-191-3p
12.	hsa-miR-30d-3p
13.	hsa-miR-365b-5p
14.	hsa-miR-551b-5p
15.	hsa-miR-221-5p
16.	hsa-miR-130b-5p
17.	hsa-miR-494-5p
18.	hsa-miR-19b-1-5p
19.	hsa-miR-3124-5p
20.	hsa-miR-1249-3p
21.	hsa-miR-1260b
22.	hsa-miR-155-3p

Table 7 presents the predicted target genes associated with selected downregulated miRNAs identified following SHG-8 treatment in U87MG glioblastoma cells. Target gene prediction analysis was performed using the miRSystem database, which generated reports of genes potentially regulated by each miRNA. The identified target genes are associated with multiple cancer-related biological processes and signaling pathways implicated in glioblastoma progression, including regulation of cell proliferation, apoptosis, angiogenesis, migration, invasion, cell cycle progression, and tumour survival.

Table 7: miRNAs with Target Genes following SHG-8 treatment.

No.	miRNAs with Predicted Target Genes Following SHG-8 Treatment	
1.	hsa-miR-421	NOX4, MTA1, CEBPB, PLK2, IDH1, CCND3, PRKD1, RASA1, SMAD1, SUZ12, MAPK14, SOX4, PDGFRA, IGF1, CDK6, XIAP, HMGB3, ROCK1, TIMP2, GOLPH3, CBX7, PDCD4, DOCK4, IGF2BP1, DYRK1A
2.	hsa-miR-92b-3p	NOX4, CEBPA, TWIST1, IDH1, LMO2, FZD10, ITGA5, RAP1B, USP28, ANGPTL2, AXL, CCNE2, GATA2, ADAM10, ARID1B, DKK3, DAB2IP, LATS2, SMAD7, LRP1B, FBXW7, PTEN, NF1, E2F3, TP63, BCL2L11, CDK6, ZEB2, SOX4, MAP2K4
3.	hsa-miR-9-5p	REST, SCRIB, CTHRC1, ITGB4, NOX4, P4HA2, FGF18, ATF1, HES1, CXCR4, WNT4, PAK4, NFKB1, MAP2K3, JUN, EFNA1, PDGFC, EPHB4, UHRF1, IGF2BP3, STMN1, ARHGEF2, ADAMTS9, TGFBI, AMBRA1
4.	hsa-miR-20a-5p	FGF4, MASTL, STK11, RHOC, MMP3, PBK, RACGAP1, PPP2R2A, PFKP, INHBA, SMAD6, ATAD2, HIF1A, JAK1, MAP3K5, MAP3K11, MAP3K12, MAPK9, E2F1, ERBB3, RRM2, FZD6, MYCN, EP300, FLT1, VEGFA, PTEN, STAT3, CCND1, RB1, CASP8, MMP2, SOX4, CDKN1A, TGFB2
5.	hsa-miR-10b-5p	PIK3CA, PTEN, LATS1, EPHA2, FLT1, SERPINE1, SMAD2, SHC1, RASAL2, MAP3K2, MAP4K4, ARHGEF11, FOXO1, HDAC4, IGF2BP2, E2F7, TFAP2A, KLF4, GATA3, BCL2L2, RB1CC1, SPOCK1, NRP2, STARD13, TEAD1
6.	hsa-miR-942-5p	CDK6, CHEK1, E2F7, SKP1, SMAD4, SMAD7, MAP3K3, CHUK, PLCB1, PRKG1, ZEB1, ZEB2, RUNX1, RREB1, SOX11, ONECUT2, SMARCA4, CREBBP, DNMT3A, ASH1L, CUL4B, FGF1, NTRK2, NTRK3, EPHA7, THBS1, LAMC1, TNS1, RNND3, PAK2, EIF4E, IGF2BP1, LIN28B
7.	hsa-miR-361-3p	SRC, YAP1, EP300, PBRM1, SMARCC1, TRIM33, BTRC, RARA, MAZ, TFAP4, ELI, HXCA10, HOXC6, PAX2, PRRX1, ARNT, BBC3, MTSS1, COL1A1, MMP24, CDC42BPB, RAP2B, DYRK1B, OGT, EIF4G1, LARP1, MED1, SMG1
8.	hsa-miR-361-3p	SRC, YAP1, EP300, PBRM1, SMARCC1, TRIM33, BTRC, RARA, MAZ, TFAP4, ELK1, HOXA10, HOXC6, PAX2, PRRX1, ARNT, BBC3, MTSS1, COL1A1, MMP24, CDC42BPB, RAP2B, DYRK1B, OGT, EIF4G1, LARP1, MED1, SMG1
9.	hsa-miR-222-3p	SMARCA4, MMP1, CDKN1C, CDKN1B, KIT, ARID1A, FOS, STMN1, AXIN2, ERBB3, PAK1, PAK2, PIK3R1, SOCS3, KDR, IGF1, THEB1, BCL2L11, APAF1, BNIP3L, ZEB2, ETS1, SOX4, BMI1, CDK6, TET2, SETD7, CBL, CXCL12, TIAM1, RECK, DICER1, SIRT1, GRB10, IGF2BP2, SMAD5, EPB41L5, ILF3, NCOR2
10.	hsa-miR-184	SPHK2, INPPL1, SND1, ARHGEF2, TNK2, SEPT9, CSF1, FZD1, BCL2L1, PRKCB, STIM1, NFATC2, FOSB, EPAS1, AKT2, RUNX1, SMAD5, EPB41L5, ILF3, NCOR2
11.	hsa-miR-941	PTPRS, ST5, GAB1, TAOK1, RASL10B, GRB2, PPP3R1, NFIX, SUV420H2
12.	hsa-miR-10a-5p	PIK3CA, PTEN, LATS1, EPHA2, FLT1, CASP8, DAPK2, SERPINE1, RASAL2, CRK, DVL3, BCL6, IGF2BP2, TIAM1, SMAD2, MAP3K7, MAP4K4, EPHA4, BCL2L11, HDAC4, FOXO1, E2F3, NFAT5, NRP2, STARD13, TEAD1
13.	hsa-miR-128-3p	EGFR, MET, PDGFRA, PIK3R1, NF1, RB1, CASP8, APAF1, YAP1, AXIN1, DVL2, WNT3A, FZD7, SFRP1, DKK2, MAPK14, MAP2K4, MAP2K7, MAP3K8, RUNX1, SMAD2, TGFB1, IGF1, IRS1, CDC42, ITGA5, ITGB8, CXCL12, CSF1, VEGFC, FGF1, FGF5, FGF7, SIRT1, HDAC4, FOXO1, FOXO4, KLF4, SET, BMI1, SUZ12, FBXW7, SPOP, PDPN, NTRK3, NRP2, RAP1B, RND3, PLK2, WEE1
14.	hsa-miR-484	PIM3, MAP3K11, ELK1, PDGFA, FOXO4, E2F1, BRD4, MAP4K5, MAX, PTPRF, FGF1, SESN2, SOX5, DACH1, FGF7, ACVR1B, RAP2A, GRB10, YAP1, TRIM33, BCL11A, MAGI1, FZD4, HIPK1, NFIB, KLF12

In Figure 14, a Venn diagram was used to identify the common genes regulated between hsa-miR-484 and hsa-miR-942-5p following SHG-8 treatment in U87MG cells. In total, 15 common target genes were identified between both miRNAs, suggesting potential co-regulation of molecular pathways associated with glioblastoma progression. Several of the shared genes, including *FOXO4*, *MAX*, *FGF1*, and *MAGI1*, are implicated in cellular proliferation, transcriptional regulation, angiogenesis, and tumour suppressive signaling pathways.

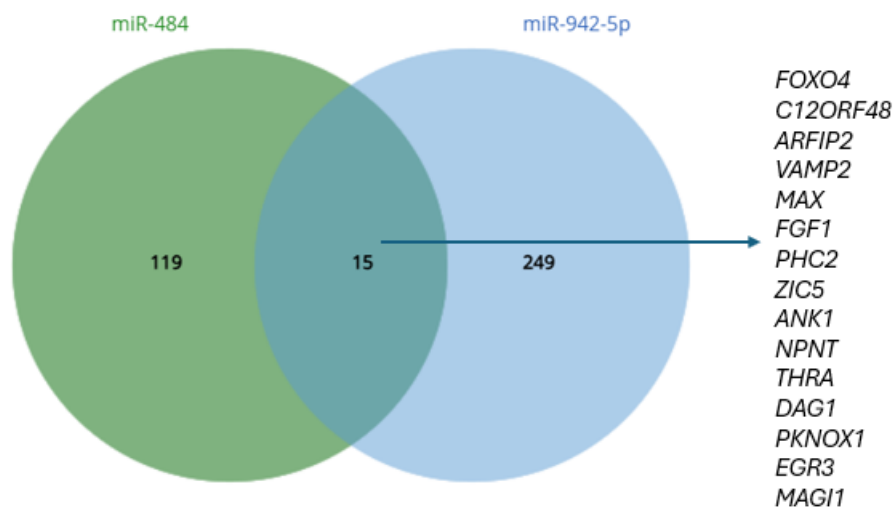


Figure 14: Venn diagram illustrating shared and unique predicted target genes between hsa-miR-484 and hsa-miR-942-5p. The Venn diagram illustrates the overlap and distribution of predicted target genes associated with hsa-miR-484 and hsa-miR-942-5p following SHG-8 treatment in U87MG cells. Bioinformatic target prediction analysis identified 119 unique target genes associated with hsa-miR-484 and 249 unique target genes associated with hsa-miR-942-5p, while 15 target genes were found to be shared between both miRNAs. Selected target genes identified within the analysis are presented alongside the diagram, including *FOXO4*, *MAX*, *FGF1*, *MAGI1*, and *EGR3*, which are implicated in pathways related to tumour progression, transcriptional regulation, cellular proliferation, and signalling.

In Figure 15 a common target between miR-484 and miR-942-5p was found to be *FOXO4* gene.

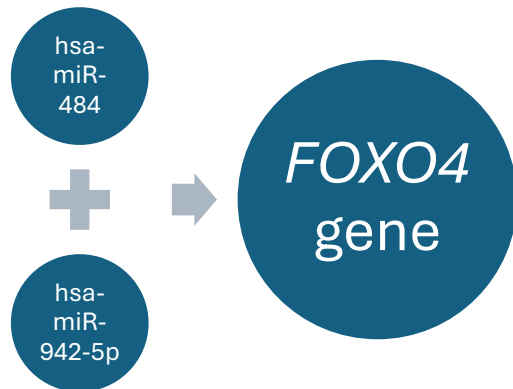


Figure 15: Co-targeting of FOXO4 by hsa-miR-484 and hsa-miR-942-5p in U87MG cells following SHG-8 treatment. The diagram illustrates the shared targeting of the gene FOXO4 by two downregulated miRNAs, hsa-miR-484 and hsa-miR-942-5p. This co-regulation indicates that FOXO4 is tightly regulated at the post-transcriptional level. FOXO4 functions as a key regulator of apoptosis and cellular stress responses in cancer cells, highlighting its importance in maintaining cellular homeostasis and tumour suppression.

In Figure 16, a Venn diagram was used to identify the common genes regulated between hsa-miR-361-3p and hsa-miR-484 following SHG-8 treatment in U87MG cells. In total, three common target genes were identified between both miRNAs, namely *YAP1*, *TRIM33*, and *ELK1*. These genes are involved in critical oncogenic signalling pathways associated with cellular proliferation, transcriptional regulation, tumour progression, and glioblastoma survival. The identification of shared target genes suggests that hsa-miR-361-3p and hsa-miR-484 may contribute to coordinated regulation of signalling networks involved in glioblastoma pathogenesis.

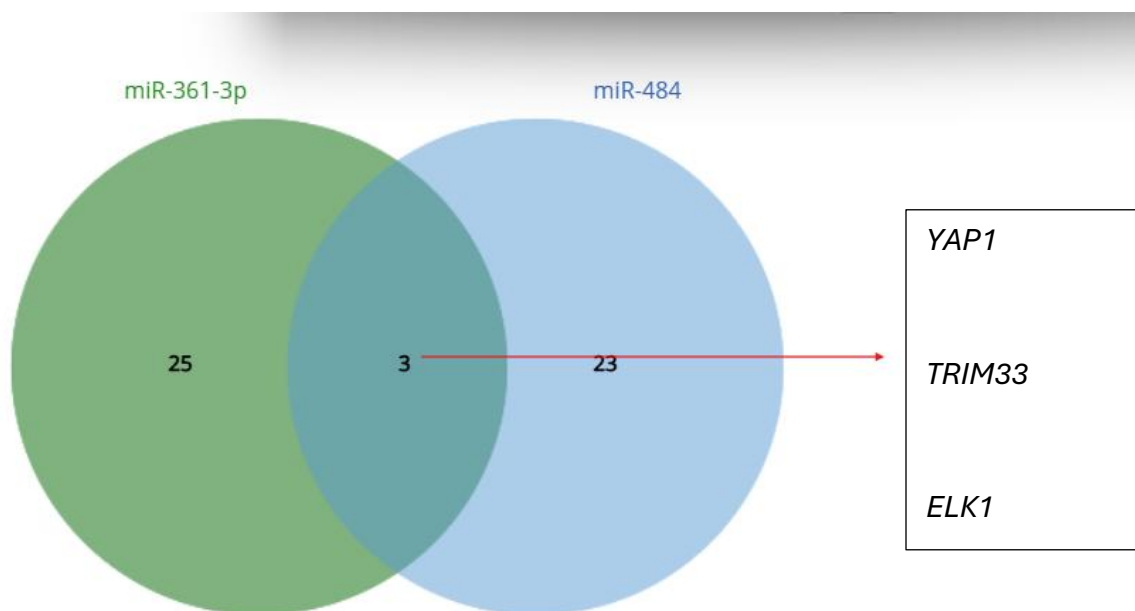


Figure 16: Venn diagram illustrating shared and unique predicted target genes between hsa-miR-361-3p and hsa-miR-484. The Venn diagram illustrates the overlap and distribution of predicted target genes associated with hsa-miR-361-3p and hsa-miR-484 following SHG-8 treatment in U87MG cells. Bioinformatic target prediction analysis identified 25 unique target genes associated with hsa-miR-361-3p, and 23 unique target genes associated with hsa-miR-484, while three target genes were identified as shared between both miRNAs. The common predicted target genes included *YAP1*, *TRIM33*, and *ELK1*, which are involved in cellular proliferation, transcriptional regulation, tumour progression, and oncogenic signalling pathways. In particular, *YAP1* is a major component

of the Hippo signalling pathway associated with glioblastoma growth and tumour survival, while *TRIM33* and *ELK1* are implicated in transcriptional regulation and MAPK-related signalling.

In Figure 17 a common target between miR-484 and miR-361-3p was found to be the *TRIM33* gene.

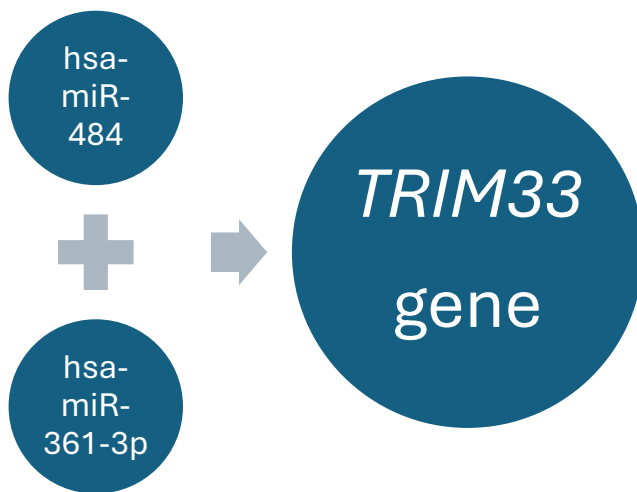


Figure 17: Co-targeting of *TRIM33* by hsa-miR-484 and hsa-miR-361-3p in U87MG cells following SHG-8 treatment. The diagram illustrates the shared targeting of the gene *TRIM33* by two downregulated miRNAs, hsa-miR-484 and hsa-miR-361-3p. This co-regulation suggests that *TRIM33* may be under coordinated post-transcriptional regulation by multiple miRNAs following SHG-8 treatment. *TRIM33* is involved in transcriptional regulation, chromatin remodelling, and modulation of TGF- β signalling pathways associated with tumour suppression and cellular differentiation. Dysregulation of *TRIM33* has been implicated in cancer progression and altered cellular signalling, highlighting its potential relevance in glioblastoma pathogenesis and tumour development.

In Figure 18, a Venn diagram was used to identify the common genes regulated between hsa-miR-10a-5p and hsa-miR-10b-5p following SHG-8 treatment in U87MG cells. In total, 15 common target genes were identified between both miRNAs, including *PTEN*, *LATS1*, *EPHA2*, *SMAD2*, *MAP4K4*, and *STARD13*. Several of the shared genes are involved in tumour suppressive pathways and oncogenic signaling networks, including the PI3K/AKT, Hippo, and TGF- β signaling pathways. The substantial overlap in predicted target genes suggests that hsa-miR-10a-5p and hsa-miR-10b-5p may cooperatively regulate multiple molecular mechanisms associated with glioblastoma proliferation, invasion, and tumour survival.

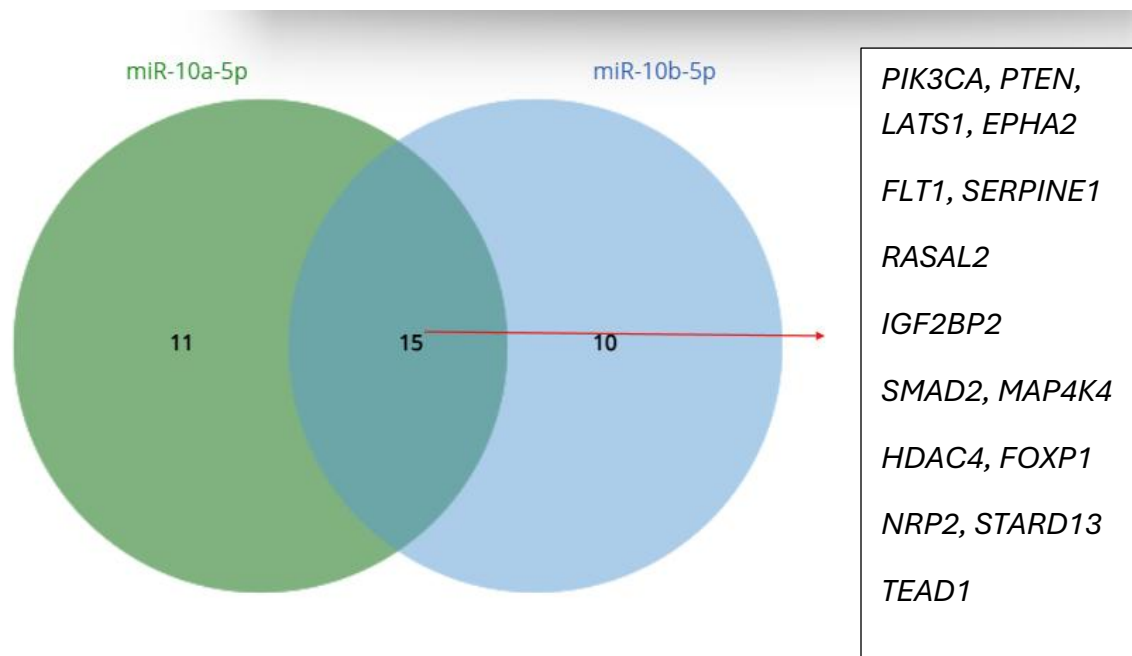


Figure 18: Venn diagram illustrating shared and unique predicted target genes between hsa-miR-10a-5p and hsa-miR-10b-5p. The Venn diagram illustrates the overlap and distribution of predicted target genes associated with hsa-miR-10a-5p and hsa-miR-10b-5p following SHG-8 treatment in U87MG cells. Bioinformatic target prediction analysis identified 11 unique target genes associated with hsa-miR-10a-5p, and 10 unique target genes associated with hsa-miR-10b-5p, while 15 target genes were found to be shared between both miRNAs. Shared target genes included *PIK3CA*, *PTEN*, *LATS1*, *EPHA2*, *FLT1*, *SERPINE1*, *RASAL2*, *IGF2BP2*, *SMAD2*, *MAP4K4*, *HDAC4*, *FOXP1*, *NRP2*, *STARD13*, and *TEAD1*. Several of these genes are involved in key oncogenic signalling pathways implicated in glioblastoma progression, including the PI3K/AKT, Hippo, TGF- β , and MAPK signalling

pathways. Notably, the shared tumour suppressor genes *PTEN* and *LATS1* are critical regulators of cellular proliferation, apoptosis, and tumour suppression.

In Figure 20, the FOXO signaling pathway was evaluated to further determine the functional significance of *FOXO4* as a predicted target gene identified through bioinformatic analysis. *FOXO4* was highlighted within the pathway owing to its established involvement in the regulation of critical cellular processes, including apoptosis, oxidative responses, autophagy, DNA damage repair, and cell cycle progression. The pathway analysis further demonstrated the extensive molecular interactions between *FOXO4*, and several key oncogenic signaling cascades implicated in glioblastoma pathogenesis, including the PI3K/AKT, MAPK, AMPK, and TGF- β signaling pathways.

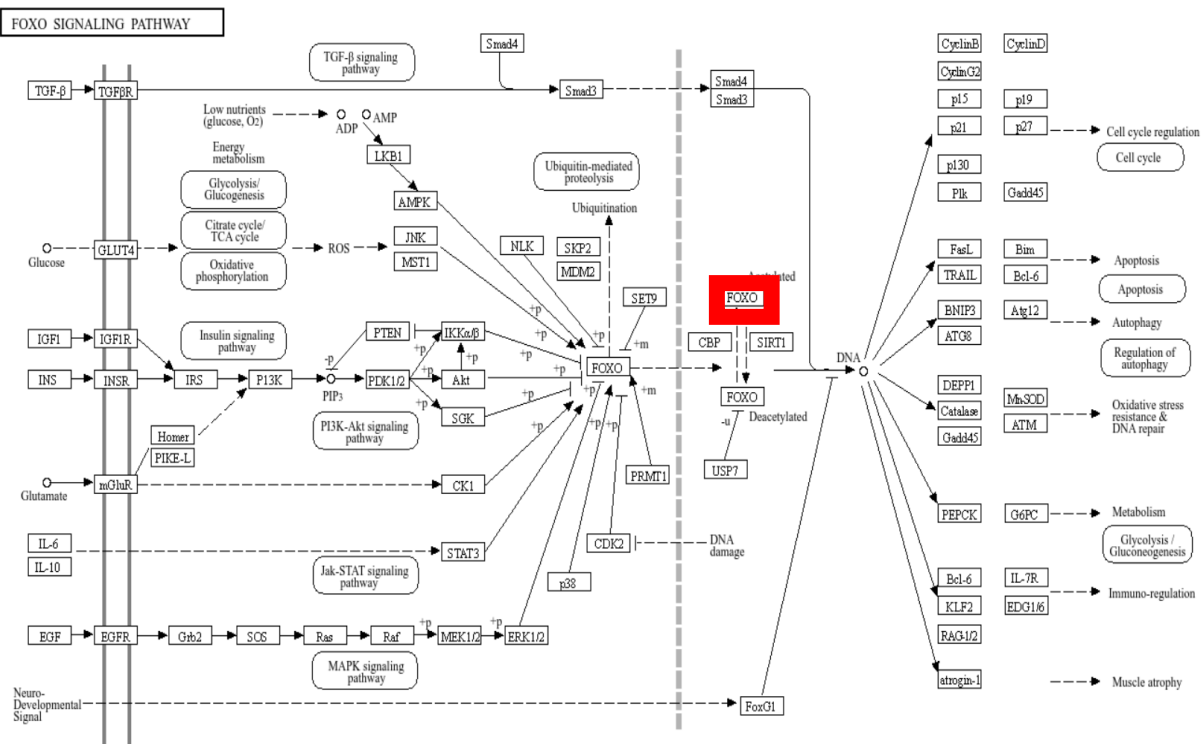


Figure 20: FOXO signalling pathway highlighting FOXO4 involvement in glioblastoma-related signalling. The figure illustrates the FOXO signalling pathway and highlights the involvement of *FOXO4* as a predicted target gene identified during bioinformatic analysis. *FOXO4* functions as a transcription factor involved in the regulation of multiple cellular processes including apoptosis, oxidative stress response, autophagy, DNA repair, metabolism, and cell cycle regulation. FOXO signalling is tightly regulated through interactions with several major oncogenic pathways, including PI3K/AKT, MAPK, AMPK, and TGF- β signalling pathways.

4. Discussion

Glioblastoma (GB) is the most aggressive and common primary malignant brain tumour in adults and remains one of the most challenging cancers to treat effectively (Ostrom et al., 2022). Classified as a grade IV astrocytoma by the World Health Organization, glioblastoma is characterised by rapid cellular proliferation, extensive angiogenesis, necrotic regions, and highly invasive behaviour (Louis et al., 2021). These biological features result in poor clinical prognosis and resistance to treatment. The symptoms associated with GB significantly impacts patient quality of life and often worsens rapidly as the disease progresses (Tan et al., 2020). From an epidemiological perspective, glioblastoma accounts for approximately 15% of all primary brain tumours and nearly half of all malignant brain tumours (Ostrom et al., 2022). The incidence rate is estimated at 3–5 cases per 100,000 individuals per year, with a slightly higher prevalence in males and in older adults, particularly those over the age of 60 (Tamimi et al., 2017). Despite advances in neurosurgery, radiotherapy, and chemotherapy, the prognosis for glioblastoma patients remains extremely poor. Median survival following diagnosis is approximately 12–15 months, and the five-year survival rate is less than 5% (Stupp et al., 2005). Recurrence is almost inevitable, and once the tumour recurs, treatment options become even more limited, further reducing survival prospects (Weller et al., 2021). The poor outcomes associated with GB are driven by several key factors. One of the most significant challenges is the highly infiltrative nature of the tumour. Glioblastoma cells invade surrounding brain tissue, making complete surgical resection virtually impossible without causing severe neurological damage (Lathia et al., 2015). Even when maximal safe resection is achieved, residual tumour cells remain and contribute to recurrence. Additionally, the blood–brain barrier (BBB) acts as a major obstacle to effective drug delivery, limiting the ability of many chemotherapeutic agents to reach therapeutic concentrations within the tumour (Pardridge et al., 2012). GB is also characterised by substantial genetic and molecular heterogeneity, which contributes to therapeutic resistance and tumour adaptability (Verhaak et al., 2010). Furthermore, the tumour microenvironment, including hypoxic regions, abnormal vasculature, and immune suppression, plays a critical role in promoting tumour growth and resistance to treatment (Quail et al., 2017). Current treatment strategies for GB, involve a multimodal approach. The standard of care includes maximal safe surgical resection followed by radiotherapy and chemotherapy, most commonly with the alkylating agent temozolomide (Stupp et al., 2005). In some cases, alternative chemotherapeutic agents such as cisplatin, a platinum-based drug, are also used due to their cytotoxic properties (Dasari et al., 2014). However, these treatments are associated with significant limitations. Surgical resection cannot eliminate all tumour cells due to their invasive nature. Radiotherapy can damage healthy brain tissue

and lead to long-term cognitive impairment (Greene-Schloesser et al., 2012). Chemotherapy is often limited by systemic toxicity, poor BBB penetration, and the development of resistance (Lee et al., 2016). As a result, these conventional therapies offer only modest improvements in survival and are rarely curative. This underscores the urgent need for novel therapeutic approaches that can overcome these barriers and improve patient outcomes. In recent years, personalised medicine has emerged as a promising strategy in oncology. This approach focuses on tailoring treatments to individual patients based on their genetic, molecular, and clinical characteristics (Ashley et al., 2016). Personalised medicine aims to maximise therapeutic efficacy while minimising adverse effects, representing a shift away from the traditional “one-size-fits-all” approach. Within this context, innovative technologies such as three-dimensional (3D) printing have gained considerable attention. 3D printing, also known as additive manufacturing, involves the layer-by-layer construction of objects based on digital designs (Javaid et al., 2019). This technology allows for precise control over the structure, composition, and properties of the final product, making it highly suitable for biomedical applications (Ngo et al., 2018). In the pharmaceutical industry, 3D printing offers significant advantages for drug delivery. It enables the fabrication of customised dosage forms with controlled drug release profiles, tailored geometries, and patient-specific dosing (Jamróz et al., 2018). This level of precision is particularly valuable in cancer treatment, where targeted and localised drug delivery can improve therapeutic outcomes and reduce systemic toxicity (Ventola et al., 2014). 3D-printed drug-loaded patches represent an innovative approach to delivering chemotherapeutic agents directly to the tumour site. Such systems can provide sustained drug release, maintain therapeutic concentrations over time, and potentially bypass the limitations imposed by the BBB (Neufeld et al., 2021). Furthermore, the use of biocompatible hydrogel materials enhances the feasibility of these systems for clinical application (Li et al., 2020). The present study investigated the potential of 3D-printed hydrogel-based patches loaded with the investigational compound SHG-8 as a novel therapeutic approach for glioblastoma. The hydrogel matrix, composed of hydroxypropyl methylcellulose (HPMC) and sodium carboxymethylcellulose (NaCMC), was selected due to its biocompatibility, swelling capacity, and ability to facilitate controlled drug release (Conti et al., 2007). Thermogravimetric analysis (TGA) was conducted to assess the thermal behaviour and stability of SHG-8, an important consideration for its potential use in pharmaceutical formulation, processing, storage, and drug delivery applications. The TGA profile indicated that SHG-8 remained stable up to approximately 190 °C, exhibiting minimal mass loss throughout this temperature range. This suggests that the compound is unlikely to experience significant heat-induced degradation under normal laboratory conditions or physiological environments. A gradual decrease in mass was observed at higher temperatures, corresponding to the initiation of thermal decomposition. These results

indicate that substantial structural degradation of SHG-8 would occur only when exposed to temperatures exceeding 190 °C. Such thermal resilience is advantageous, as it implies that routine manufacturing and formulation procedures, including solvent removal and moderate heating processes, are unlikely to adversely affect the compound's chemical structure. The observed stability is also particularly relevant to the development of the 3D-printed hydrogel patch, since drug loading, storage, and localised administration are not expected to involve temperatures approaching the decomposition threshold. Overall, the TGA findings demonstrate that SHG-8 possesses favourable thermal stability characteristics, supporting its continued investigation as a potential therapeutic agent (Almanghrabi, 2023). The effects of SHG-8-loaded patches on U87MG glioblastoma cells were evaluated using three key assays: the MTT assay to assess cell viability, the scratch (wound healing) assay to evaluate cell migration, and the colony formation assay to determine long-term proliferative capacity. The MTT assay results demonstrated that SHG-8 delivered via the 3D-printed patches induced a dose-dependent reduction in cell viability. Quantitative analysis demonstrated differences in viability between the control and treatment groups, indicating that SHG-8 patch treatment reduced U87MG cell survival in a concentration-dependent manner. The viability data obtained were subsequently used for graphical representation and IC50 estimation analyses. As shown from the results, the percentage cell viability values were obtained from three independent biological replicates (N1, N2, and N3) following treatment with DMSO control, 200 µM cisplatin, and SHG-8 patch concentrations of 25 µM, 50 µM, and 100 µM. The DMSO control group showed the highest average viability at 100%, indicating that the control did not induce any cytotoxicity. Treatment with 200 µM cisplatin resulted in an average viability of 61%, demonstrating reduced cell survival relative to the DMSO control. Treatment with the SHG-8 patch resulted in lower variability across all tested concentrations. The 25 µM SHG-8 patch treatment resulted in an average cell viability of 49%, while treatment with 50 µM also produced an average viability of 49%. The 100 µM SHG-8 patch treatment exhibited the lowest average viability at 42%, indicating the strongest cytotoxic effect among all SHG-8 treatment groups. These findings suggest that increasing SHG-8 patch concentration enhanced the reduction in U87MG cell viability, supporting the anti-proliferative activity of the formulation. However, significant reductions in cell viability were observed in SHG-8 patch-treated groups when compared with the DMSO control, particularly at higher concentrations. The graphical trend demonstrated an overall decline in viability following SHG-8 patch treatment, supporting the cytotoxic activity of the formulation against U87MG glioblastoma cells. Following analysis of cell viability across the SHG-8 patch treatment concentrations, the dose-response data generated from the MTT assay were used to estimate IC50 values for each independent biological replicate. The IC50 value represents the concentration required to reduce cell viability by 50% relative to the control condition and provides an indication of treatment

potency (Aykul et al., 2017). As shown from the results, replicate N1 produced an estimated IC₅₀ value of 50.0 μ M, while replicate N2 generated the highest IC₅₀ value at 62.7 μ M. Replicate N3 demonstrated the lowest IC₅₀ value of 41.7 μ M. The calculated mean IC₅₀ value across all biological replicates was 51.4 μ M. This finding indicates that approximately 51 μ M of SHG-8 was required to reduce U87MG cell viability by 50% under the experimental conditions used in this study. Collectively, these findings suggest that the SHG-8 patch effectively impaired U87MG glioblastoma cell survival and proliferation, highlighting its potential as a localized anti-cancer therapeutic system. The sustained reduction in viability observed following treatment may indicate disruption of essential cellular processes involved in tumor cell metabolism, growth, and survival (Neophytou et al., 2021). Importantly, the ability of the SHG-8 patch to reduce glioblastoma cell viability at micromolar concentrations supports the feasibility of utilizing this formulation as a targeted drug-delivery platform for glioblastoma treatment. Such an approach may provide advantages over conventional systemic chemotherapy by enabling localized drug exposure at the tumor site while potentially reducing systemic toxicity and overcoming limitations associated with blood-brain barrier penetration. Cisplatin and temozolomide are among the most widely investigated chemotherapeutic agents in glioblastoma treatment due to their ability to interfere with DNA replication and induce tumour cell death. Cisplatin is a platinum-based chemotherapeutic drug that exerts its anti-cancer activity primarily through the formation of DNA crosslinks, leading to disruption of DNA replication and transcription, activation of DNA damage responses, and induction of apoptosis in rapidly proliferating tumour cells (Romani, 2022) (Dasari et al., 2014). Similarly, temozolomide (TMZ), an oral second-generation alkylating agent, induces cytotoxicity through methylation of DNA at the O6 and N7 positions of guanine residues, resulting in DNA damage, cell cycle arrest, and apoptosis (Friedman et al., 2000). Temozolomide remains the current standard chemotherapeutic agent used in glioblastoma management due to its ability to cross the blood-brain barrier and demonstrate activity against high-grade gliomas (Friedman et al., 2000). However, despite its clinical significance, resistance to temozolomide is common, and glioblastoma cells frequently exhibit reduced sensitivity to treatment, contributing to poor long-term therapeutic outcomes (Poon et al., 2021). In the present study, cisplatin treatment reduced U87MG cell viability and colony formation relative to the DMSO control, confirming its established cytotoxic activity against glioblastoma cells. Nevertheless, SHG-8-loaded patch treatment demonstrated a greater inhibitory effect on both short-term metabolic activity and long-term clonogenic survival under the experimental conditions tested. While cisplatin-treated cells retained measurable viability and colony-forming capacity, SHG-8 patch-treated groups, particularly at higher concentrations, exhibited substantially lower viability percentages together with near-complete suppression of colony formation. These findings suggest that the SHG-8 patch may exert enhanced anti-cancer

activity through a combination of direct cytotoxic effects and local microenvironmental modulation. Importantly, comparison with current glioblastoma chemotherapeutics further highlights the significance of the observed findings. Previous systematic evaluation of malignant glioma cell lines demonstrated that U87 glioblastoma cells often exhibit relatively high IC₅₀ values following temozolomide treatment, indicating substantial resistance and reduced therapeutic sensitivity *in vitro* (Poon et al., 2021). In contrast, the SHG-8 patch demonstrated measurable cytotoxicity at substantially lower micromolar concentrations, with an estimated mean IC₅₀ value of 51.4 μ M in U87MG cells. The ability of SHG-8 treatment to reduce both viability and clonogenic survival at comparatively moderate concentrations may therefore indicate promising anti-glioblastoma potential relative to conventional chemotherapeutic approaches. The observed reduction in colony formation following SHG-8 treatment is particularly important in the context of GB recurrence. GB is characterised by highly invasive and treatment-resistant tumour cell populations capable of surviving conventional therapy and regenerating tumour growth following treatment (Mattei et al., 2021). While cisplatin and temozolomide can effectively induce DNA damage and apoptosis, surviving glioblastoma cells frequently retain sufficient proliferative capacity to re-establish tumour colonies and contribute to recurrence (Friedman et al., 2000). In the current study, the substantial suppression of clonogenic survival observed following SHG-8 patch treatment suggests that the formulation may impair not only short-term cellular metabolism but also the long-term reproductive and regenerative capacity of glioblastoma cells. Furthermore, conventional systemic chemotherapy is often associated with major limitations including systemic toxicity, nephrotoxicity, gastrointestinal toxicity, myelosuppression, poor tumour selectivity, and restricted drug accumulation within the brain due to the blood-brain barrier (Romani et al., 2022) (Dasari et al., 2014). The localised drug-delivery approach utilised by the SHG-8-loaded hydrogel patch may therefore offer several potential therapeutic advantages, including sustained local drug exposure, reduced systemic toxicity, and improved targeting of tumour tissue (Norouzi et al., 2016). Collectively, these findings support the potential therapeutic value of SHG-8-loaded patches as an alternative or adjunctive localised treatment strategy for glioblastoma, particularly in overcoming limitations associated with conventional chemotherapeutic agents such as cisplatin and temozolomide. Colony formation analysis was performed to evaluate the long-term clonogenic survival and proliferative capacity of U87MG glioblastoma cells following treatment with SHG-8-loaded patches. Unlike the MTT assay, which primarily measures short-term metabolic activity, the colony formation assay assesses the ability of single surviving cells to proliferate and form colonies over an extended period (Franken et al., 2006). Quantitative colony counts obtained from three independent biological replicates were analysed and compared between the DMSO control, 200 μ M cisplatin-treated group, 40 μ M SHG-8 patch-treated group, and 60 μ M SHG-8 patch-treated group. As shown from the

results, the DMSO control group consistently demonstrated the highest colony counts across all biological replicates, indicating strong clonogenic survival and normal proliferative activity in untreated cells. Average colony counts of 94.3, 26.3, and 54.0 were recorded in biological replicates N1, N2, and N3, respectively. In comparison, treatment with 200 μ M cisplatin reduced colony formation, producing average colony counts of 36.3, 7.33, and 18.0 across replicates N1, N2, and N3. These findings indicate that cisplatin exerted a suppressive effect on glioblastoma cell proliferation relative to the DMSO control. Treatment with SHG-8-loaded patches resulted in a substantially greater reduction in colony formation compared with both the DMSO control and cisplatin-treated groups. The 40 μ M SHG-8 patch treatment produced average colony counts of 13.0, 3.66, and 4.33 across biological replicates N1, N2, and N3, respectively. A further reduction in colony formation was observed following treatment with the 60 μ M SHG-8 patch, which generated extremely low average colony counts of 0.33, 1.0, and 0.66 across the three biological replicates. These findings demonstrate a pronounced inhibitory effect of SHG-8 patch treatment on the clonogenic capacity of U87MG glioblastoma cells. When colony counts from all biological replicates were combined, the DMSO control group demonstrated an overall average colony count of 58.2, whereas the 200 μ M cisplatin-treated group exhibited a reduced average colony count of 20.54. In contrast, treatment with the 40 μ M SHG-8 patch produced a markedly lower average colony count of 7.00, while the 60 μ M SHG-8 patch treatment resulted in the lowest overall average colony count of 0.66. These results indicate that SHG-8 patch treatment produced a substantially stronger inhibitory effect on colony formation than cisplatin under the experimental conditions tested. As shown graphically in the results, one-way ANOVA analysis demonstrated a statistically significant difference among treatment group means ($p = 0.0224$). Multiple comparison analysis revealed no significant difference between the DMSO control group and the 200 μ M cisplatin-treated group. However, statistically significant reductions in colony formation were identified in both the 40 μ M SHG-8 patch-treated group and the 60 μ M SHG-8 patch-treated group relative to the DMSO control group ($p < 0.05$). Additionally, colony counts observed following 40 μ M SHG-8 patch treatment remained greater than those observed following 60 μ M SHG-8 patch treatment, further supporting a concentration-dependent inhibitory effect on clonogenic survival. The reduction in colony formation observed following SHG-8 patch treatment suggests that the formulation not only reduced short-term cellular metabolic activity, as demonstrated in the MTT assay, but also impaired the proliferative potential of surviving glioblastoma cells. This finding is particularly important because colony formation assays evaluate the ability of cancer cells to recover, divide, and establish new tumour populations following treatment (Franken et al., 2006). The near-complete suppression of colony formation observed at 60 μ M indicates that SHG-8 treatment severely compromised the capacity of U87MG cells to survive and proliferate over time. Overall, these findings

demonstrate that the SHG-8-loaded patch possesses strong anti-clonogenic activity against U87MG glioblastoma cells. The substantial reduction in colony counts, particularly at 60 μ M, suggests that the formulation may interfere with critical cellular processes involved in tumour cell survival, proliferation, and regenerative capacity. Furthermore, the stronger inhibitory effect observed in SHG-8-treated groups relative to cisplatin-treated cells highlights the potential therapeutic value of the SHG-8 patch as a localised anti-cancer treatment strategy for glioblastoma. The ability to suppress both short-term viability and long-term clonogenic survival supports the potential application of SHG-8-loaded patches as an effective targeted drug-delivery platform for glioblastoma therapy. However, it is important to recognise that the observed cytotoxic and anti-clonogenic effects may not be solely attributable to the pharmacological activity of SHG-8. The physical and chemical properties of the 3D-printed hydrogel patch itself may also have contributed to the reduction in U87MG cell viability and colony formation. The hydrogel matrix, composed primarily of hydroxypropyl methylcellulose (HPMC), sodium carboxymethyl cellulose (NaCMC), glycerol, and water, exhibits highly hydrophilic characteristics that promote rapid swelling when exposed to aqueous environments (Li et al., 2020). Following placement into the culture wells, the patch likely absorbed surrounding media and expanded over or in close proximity to the cell monolayer, thereby modifying the local cellular microenvironment. One important consequence of hydrogel swelling may have been the restriction of oxygen diffusion to the underlying glioblastoma cells (Mavris et al., 2021). U87MG cells are highly metabolically active and rely heavily on oxygen-dependent mitochondrial respiration to maintain cellular proliferation, ATP production, and survival (Ledur et al., 2017). The swollen polymer matrix may therefore have created a localised hypoxic environment by partially obstructing oxygen transfer from the culture medium and atmospheric interface to the adherent cells beneath the patch. Similar diffusion-related limitations have previously been reported in hydrogel-based culture systems and polymeric biomaterials, where increased matrix density and swelling reduce oxygen permeability and alter cellular metabolic activity (Cervellati et al., 2013) (Lovett et al., 2009). The development of local hypoxia is particularly relevant in the context of the MTT assay, as MTT reduction depends directly on mitochondrial dehydrogenase enzyme activity (Ghasemi et al., 2021). Reduced oxygen availability can impair oxidative phosphorylation and mitochondrial electron transport, leading to decreased conversion of MTT into insoluble formazan crystals and consequently lower apparent cell viability values (Semenza et al., 2012). Therefore, the reduction in MTT signal observed following SHG-8 patch treatment may reflect not only direct drug-induced cytotoxicity but also suppression of mitochondrial metabolic activity caused by oxygen deprivation. In addition to impairing mitochondrial respiration, hypoxic stress can significantly influence tumour cell behaviour and therapeutic sensitivity. Hypoxia is known to activate multiple cellular stress-response pathways, including hypoxia-

inducible factor (HIF)-mediated signalling, oxidative stress responses, autophagy, and apoptosis-related mechanisms (Wilson et al., 2011). Under certain conditions, hypoxia can sensitise tumour cells to cytotoxic compounds by increasing metabolic stress and reducing the ability of cells to recover from drug-induced damage (Rankin et al., 2016). Consequently, the swollen hydrogel matrix may have amplified the cytotoxic activity of SHG-8 by simultaneously delivering the drug while generating a low-oxygen microenvironment that enhanced tumour cell susceptibility to treatment. The observed correlation between reduced colour intensity during MTT analysis and decreased cell viability further supports the biological significance of these findings. Since MTT reduction is dependent on mitochondrial enzyme activity, diminished formazan formation likely reflected both reduced viable cell numbers and impaired mitochondrial metabolism induced by hypoxic stress. Similarly, the substantial reduction in colony formation observed following SHG-8 patch treatment suggests that prolonged exposure to both the chemotherapeutic compound and altered microenvironmental conditions impaired the long-term proliferative and clonogenic capacity of surviving glioblastoma cells. The near-complete suppression of colony formation observed at higher SHG-8 patch concentrations indicates that surviving cells were unable to effectively recover and re-establish proliferative colonies following treatment exposure. Although the findings demonstrate encouraging anti-cancer activity, several limitations of the study should be acknowledged. Firstly, the experiments were conducted exclusively *in vitro* using the U87MG glioblastoma cell line. While U87MG cells are widely utilised in glioblastoma research, they do not fully replicate the complex biological heterogeneity, invasive behaviour, stromal interactions, and immune components observed in patient-derived glioblastoma tumours *in vivo* (Allen et al., 2016) (Huse et al., 2010). As a result, the observed responses may not accurately predict therapeutic performance under physiological conditions. Secondly, separating the direct pharmacological effects of SHG-8 from the physical effects induced by the hydrogel matrix remains challenging. The swelling behaviour of the patch may have altered oxygen diffusion, nutrient transport, waste accumulation, osmotic balance, and physical interactions with the cell monolayer, all of which could independently influence cellular viability and proliferation (Mavris et al., 2021). Consequently, the enhanced cytotoxicity observed with SHG-8-loaded patches likely reflects a combined effect of drug activity and microenvironmental modulation produced by the hydrogel system. Another important limitation relates to experimental reproducibility and sample size. Furthermore, the current study primarily focused on short-term cytotoxicity and clonogenic survival assays, providing limited insight into the long-term biological behaviour, stability, biodegradation, and sustained therapeutic effects of the 3D-printed hydrogel patches. Importantly, the drug release kinetics of the SHG-8-loaded patches were not comprehensively characterised. Controlled drug release represents a critical parameter in hydrogel-based delivery systems,

as polymer swelling, degradation, and diffusion properties directly influence therapeutic exposure and efficacy (Siepmann et al., 2011). Without detailed release-profile analysis, the precise concentration of SHG-8 delivered to the cells over time remains uncertain. Consequently, the exact relationship between patch concentration, local drug exposure, and biological response could not be fully established. Future studies should therefore incorporate controlled drug release investigations, pharmacokinetic analysis, and quantitative assessment of oxygen concentration within the culture system. Additional evaluation using multiple glioblastoma cell lines, patient-derived tumour models and in vivo animal studies would further improve the translational relevance of the findings. Investigation of hypoxia-related biomarkers, including HIF-1 α expression, reactive oxygen species generation, and apoptosis-associated proteins, would also help clarify the extent to which hypoxia contributed to the observed cytotoxic effects (Greijer et al., 2004). Collectively, while the current findings demonstrate promising anti-cancer potential for SHG-8-loaded hydrogel patches, further optimisation and mechanistic investigation will be required before the system can be considered suitable for future clinical application. Small RNA (sRNA) sequencing analysis was performed to identify differentially expressed microRNAs (miRNAs) in U87MG glioblastoma cells following treatment with the SHG-8 drug. Sequencing analysis identified multiple miRNAs exhibiting altered expression profiles relative to untreated control cells. Differential expression analysis demonstrated that several miRNAs were significantly downregulated following SHG-8 treatment and were subsequently selected for further bioinformatic investigation. The resulting expression profiles provided insight into post-transcriptional regulatory mechanisms potentially associated with the anti-cancer effects observed during cytotoxicity and colony formation assays. As shown from the results, multiple downregulated miRNAs identified through sRNA sequencing analysis were associated with genes involved in critical glioblastoma-related signalling pathways. Bioinformatic analysis demonstrated that predicted target genes were linked to several molecular pathways implicated in tumour progression, proliferation, invasion, apoptosis, angiogenesis, and treatment resistance. These included the PI3K/AKT, MAPK, Wnt/ β -catenin, FOXO, Hippo, and TGF- β signalling pathways, all of which play established roles in glioblastoma pathogenesis and tumour survival (Khabibov et al., 2022). Several tumour suppressor genes and regulatory genes, including *PTEN*, *FOXO4*, *YAP1*, *LATS1*, and *TRIM33*, were identified among the predicted targets, suggesting that SHG-8 treatment may influence complex post-transcriptional regulatory networks involved in glioblastoma cell behaviour (Wu et al., 2022). Comparative bioinformatic analysis using Venn diagrams identified overlapping target genes shared between selected downregulated miRNAs. As shown from the results, analysis of hsa-miR-484 and hsa-miR-942-5p identified 15 common target genes, suggesting potential co-regulation of shared signalling pathways. Several overlapping genes, including *FOXO4* and *MAGI1*, are associated with cellular

proliferation, transcriptional regulation, angiogenesis, apoptosis, and tumour suppressive signalling mechanisms (Farhan et al., 2020) (Worthmuller et al., 2021). The identification of multiple common target genes suggests that SHG-8 treatment may alter coordinated miRNA regulatory networks rather than affecting isolated signalling pathways individually. Further analysis demonstrated that *FOXO4* represented a common target gene regulated by both hsa-miR-484 and hsa-miR-942-5p. *FOXO4* belongs to the FOXO family of transcription factors, which are critically involved in apoptosis, oxidative stress regulation, autophagy, DNA repair, and cell cycle arrest (Zhang et al., 2011). Co-targeting of *FOXO4* by multiple downregulated miRNAs suggests that SHG-8 treatment may influence pathways associated with tumour suppression and cellular stress responses in glioblastoma cells. Since FOXO signalling is closely linked to PI3K/AKT pathway activity, alterations in miRNA-mediated regulation of *FOXO4* may contribute to the reduced viability and proliferative capacity observed following SHG-8 treatment. Additional overlap analysis identified three common target genes shared between hsa-miR-361-3p and hsa-miR-484, namely *YAP1*, *TRIM33*, and *ELK1*. These genes are associated with transcriptional regulation, chromatin remodelling, Hippo signalling, tumour progression, and cellular proliferation (Paul et al., 2022). *YAP1* functions as a major downstream effector of the Hippo pathway and has been implicated in glioblastoma stemness, tumour growth, and treatment resistance (Orr et al., 2011). Similarly, *TRIM33* is involved in modulation of TGF- β signalling and transcriptional regulation (Lee et al., 2022). The identification of shared target genes between multiple miRNAs further supports the presence of coordinated post-transcriptional regulatory mechanisms following SHG-8 treatment. As shown from the pathway mapping results, *FOXO4* was positioned within a highly interconnected regulatory network involving PI3K/AKT, MAPK, AMPK, and TGF- β signalling pathways. FOXO transcription factors function as critical regulators of apoptosis, oxidative stress resistance, autophagy, metabolic homeostasis, and DNA damage repair (Lam et al., 2013). Dysregulation of FOXO signalling has been strongly associated with tumour progression and therapeutic resistance in glioblastoma and other malignancies (Tabnak et al., 2023). Therefore, alterations in miRNA-mediated regulation of *FOXO4* following SHG-8 treatment may contribute to the observed anti-proliferative and cytotoxic effects by influencing apoptosis-associated and stress-response signalling pathways (Urbanek et al., 2016). Collectively, the bioinformatic findings suggest that SHG-8 treatment induced widespread alterations in miRNA expression profiles associated with multiple oncogenic and tumour suppressive signalling pathways relevant to glioblastoma biology. The identification of shared target genes and overlapping signalling networks indicates that SHG-8 may exert anti-cancer effects through coordinated post-transcriptional modulation of pathways involved in proliferation, survival, apoptosis, invasion, and cellular stress regulation. Importantly, several identified targets, including *FOXO4*, *PTEN* and *TRIM33*, are recognised regulators of glioblastoma progression and

therapeutic response, further supporting the biological relevance of the sequencing findings. The bioinformatic analysis revealed that a large proportion of the predicted target genes identified following SHG-8 treatment were associated with major oncogenic signalling pathways involved in glioblastoma progression and tumour survival. Several genes linked to the PI3K/AKT signalling pathway, including *PIK3CA*, *PIK3R1*, *AKT2*, and *PTEN*, were identified, suggesting that SHG-8-induced miRNA dysregulation may influence cellular proliferation, metabolism, survival, and resistance to apoptosis, while dysregulation of the PI3K/AKT pathway is one of the most common molecular abnormalities observed in glioblastoma and contributes significantly to tumour aggressiveness, uncontrolled growth, and therapeutic resistance (Molinari, 2010). Additionally, multiple components of the MAPK signalling pathway, including members of the *MAP3K* family, *MAP2K4*, and *MAPK14*, were identified, indicating potential modulation of pathways controlling cellular stress responses, proliferation, differentiation, and tumour progression (Dhillon et al., 2007). Genes associated with Wnt/ β -catenin signalling, including *AXIN1*, *DVL2/3*, and *FZD* receptors, together with TGF- β pathway components such as *SMAD* family members and *TGFBR1/2*, were also identified, while these signalling pathways are critically involved in glioblastoma stemness, epithelial-mesenchymal transition (EMT), invasion, and maintenance of cancer stem cell populations (Kahlert et al., 2012). The identification of these targets suggests that SHG-8 treatment may influence multiple interconnected signalling cascades involved in glioblastoma pathogenesis rather than acting through a single isolated molecular pathway. Several identified target genes were also associated with regulation of the cell cycle and tumour proliferation. These included *CDK6*, *CCND1*, *RB1*, and members of the *E2F* transcription factor family, all of which are central regulators of G1-S phase progression and DNA replication, while dysregulation of these proteins is strongly associated with uncontrolled tumour proliferation and increased clonogenic potential in glioblastoma (Cancer Genome Atlas Research Network, 2008). The analysis additionally identified numerous genes involved in apoptosis and cell survival pathways, including members of the *BCL2* family (*BCL2L1*, *BCL2L2*, and *BCL2L11*), together with *CASP8* and *APAF1*, which regulate intrinsic and extrinsic apoptotic signalling mechanisms and are frequently dysregulated in glioblastoma cells to promote survival and resistance to therapy (Fulda et al., 2009). The identification of FOXO transcription factors and *SIRT1* further suggested involvement of oxidative stress responses, DNA repair pathways, autophagy, and apoptosis-associated signalling. FOXO proteins are recognised tumour suppressors that regulate cell cycle arrest, oxidative stress adaptation, and apoptosis, while loss of FOXO activity has been associated with glioblastoma progression and poor prognosis (Zhang et al., 2011). A significant number of identified targets were also associated with tumour invasion, migration, and tumour microenvironment interactions. These included extracellular matrix-related genes such as *COL1A1*, *THBS1*, and members of the *MMP* family, together with

cytoskeletal regulators including *CDC42*, Rho GTPases, and PAK kinases. These genes are essential regulators of cell adhesion, migration, extracellular matrix remodelling, and invasive tumour behaviour (Nakada et al., 2007). Additionally, genes involved in angiogenesis and adaptation to hypoxic conditions, including *VEGFA*, *KDR*, *FLT1*, *HIF1A*, and *EPAS1*, were identified, while glioblastoma is characterised by extensive vascular proliferation and hypoxic tumour regions that promote tumour survival and resistance to therapy (Jensen et al., 2009). Therefore, modulation of these pathways may indicate that SHG-8 influences tumour vascularisation and hypoxia-associated survival mechanisms. Importantly, several genes were identified as shared targets regulated by multiple miRNAs, including *TRIM33*, *ELK1*, *BTRC*, *LARP1*, and *FOXO4*. These genes represent key regulatory nodes within interconnected cellular signalling networks. *TRIM33* functions as a tumour suppressor involved in TGF- β signalling regulation, while *ELK1* acts as a MAPK-responsive transcription factor associated with proliferation and oncogenic signalling. *BTRC* regulates components of the Wnt/ β -catenin pathway, *LARP1* is associated with mRNA translation and protein synthesis in cancer cells, and *FOXO4* plays a major role in apoptosis and cellular stress responses (Farhan et al., 2020) (Paul et al., 2022). The identification of these genes as targets of multiple miRNAs suggests the presence of coordinated post-transcriptional regulatory mechanisms, where disruption of miRNA expression may amplify downstream effects on critical biological pathways. Such multi-miRNA targeting is often indicative of genes with high functional importance in maintaining cellular homeostasis and regulating tumour progression (Olson et al., 2011). Collectively, the bioinformatic analysis suggests that SHG-8-induced downregulation of miRNAs resulted in widespread alterations in interconnected gene regulatory networks controlling proliferation, apoptosis, invasion, survival, angiogenesis, and tumour microenvironment interactions. These findings indicate that the anti-cancer effects of SHG-8 are unlikely to be mediated through a single pathway alone but instead involve coordinated modulation of multiple molecular signalling networks associated with glioblastoma progression and tumour maintenance.

References

1. Filho, A. M., Znaor, A., Sung, H., Zahwe, M., Marcos-Gragera, R., Jonine, D., et al. (2025). Cancers of the brain and central nervous system: Global patterns and trends in incidence. *Journal of Neuro-Oncology*, 172, 567–578.
2. Adams, H., Chaichana, K. L., Avendano, J., Liu, B., & Raza, S. M. (2018). Adult brain tumors: Current knowledge and advances in diagnosis and treatment. *American Journal of Pathology*, 188(5), 1230–1243.
3. Wen, P. Y., Weller, M., Lee, E. Q., Alexander, B. M., Barnholtz-Sloan, J. S., Barthel, F. P., et al. (2020). Glioblastoma in adults: A Society for Neuro-Oncology (SNO) and European Society of Neuro-Oncology (EANO) consensus review on current management and future directions. *British Journal of Cancer*, 123, 123–138.
4. van den Bent, M. J., Geurts, M., French, P. J., Smits, M., Capper, D., Bromberg, J. E. C., et al. (2023). Primary brain tumours in adults. *The Lancet*, 402(10412), 1564–1579.
5. Davis, M. E. (2016). Glioblastoma: Overview of disease and treatment. *Clinical Journal of Oncology Nursing*, 20(5 Suppl), S2–S8.
6. Grochans, S., Cybulska, A. M., Simińska, D., Korbecki, J., Kojder, K., Chlubek, D., & Baranowska-Bosiacka, I. (2022). Epidemiology of glioblastoma multiforme: Literature review. *Cancers*, 14(10), 2412.
7. Zhang, Y., Liu, X., & Wang, H. (2025). Emerging molecular mechanisms and therapeutic advances in glioblastoma. *Signal Transduction and Targeted Therapy*, 10, 115.
8. Gillespie, C. S., Bligh, E. R., Poon, M. T. C., Solomou, G., Islim, A. I., Mustafa, M. A., Rominiyi, O., Williams, S. T., Kalra, N., & Mathew, R. K. (2022). Imaging timing after glioblastoma surgery (INTERVAL-GB): Protocol for a UK and Ireland multicentre retrospective cohort study. *BMJ Open*, 12(9), e063043.
9. International Agency for Research on Cancer. (2024). *Global Cancer Observatory*.
10. Ilzerman-Korevaar, M., Snijders, T. J., De Graeff, A., Teunissen, S. C. C. M., & De Vos, F. Y. F. (2018). Prevalence of symptoms in glioma patients throughout the disease trajectory: A systematic review. *Journal of Neuro-Oncology*, 140, 485–496.
11. StatPearls Publishing. (2024). *Glioblastoma multiforme*. In *StatPearls*.
12. Tan, A. C., Ashley, D. M., López, G. Y., Malinzak, M., Friedman, H. S., & Khasraw, M. (2020). Management of glioblastoma: State of the art and future directions. *CA: A Cancer Journal for Clinicians*, 70(4), 299–312.

13. Thakkar, J. P., Dolecek, T. A., Horbinski, C., Ostrom, Q. T., Lightner, D. D., Barnholtz-Sloan, J. S., & Villano, J. L. (2014). Epidemiologic and molecular prognostic review of glioblastoma. *Cancer Epidemiology, Biomarkers & Prevention*, 23(10), 1985–1996.
14. Thenuwara, G., Curtin, J., & Tian, F. (2023). Advances in diagnostic tools and therapeutic approaches for gliomas: A comprehensive review. *Sensors*, 23(24), 9842.
15. Wang, X., Li, Y., & Chen, H. (2024). Novel therapeutic strategies in glioblastoma management. *Frontiers in Oncology*, 14, 1289345.
16. Håberg, A., Kvistad, K. A., Unsgård, G., & Haraldseth, O. (2011). Multiparametric analysis of magnetic resonance images for glioma grading and patient survival time prediction. *Acta Radiologica*, 52(3), 343–354.
17. Lee, J. H., Kim, S. Y., & Park, J. W. (2024). Advances in glioblastoma molecular targeted therapy and personalized medicine. *Neuro-Oncology Advances*, 6(1), vdae211.
18. Jain, K. K. (2018). A critical overview of targeted therapies for glioblastoma. *Frontiers in Oncology*, 8, 419.
19. Zhang, C., Nance, E. A., Mastorakos, P., Chisholm, J., Berry, S., Eberhart, C., Tyler, B., Brem, H., Suk, J. S., & Hanes, J. (2017). Convection enhanced delivery of cisplatin loaded brain penetrating nanoparticles cures malignant glioma in rats. *Journal of Controlled Release*, 263, 112–119.
20. Brodbelt, A., Greenberg, D. C., Winters, T., Williams, M., Vernon, S., & Collins, V. P. (2015). Glioblastoma in England: 2007–2011. *European Journal of Cancer*, 51, 533–542.
21. Hossain, M., Das, U., & Dimmock, J. R. (2019). Recent advances in α,β -unsaturated carbonyl compounds as mitochondrial toxins. *European Journal of Medicinal Chemistry*, 183, 111687.
22. Ebrahimi, S. O., Reisi, S., & Shareef, S. (2020). miRNAs, oxidative stress, and cancer: A comprehensive and updated review. *Journal of Cellular Physiology*, 235(11), 8812–8825.
23. Singh, R., Bhasin, G., Srivastava, R., & Geetanjali. (2016). β -Aminocarbonyl compounds: Chemistry and biological activities. *Mini-Reviews in Organic Chemistry*, 13(2), 143–153.
24. Mardani, Z., Kazemshoar Duzdüzani, R., Moeini, K., Hajabbas Farshchi, A., Carpenter-Warren, C., Slawin, A. M. Z., & Woollins, J. D. (2018). Anticancer activities of a β amino alcohol ligand and nanoparticles of its copper(II) and zinc(II) complexes evaluated by experimental and theoretical methods. *RSC Advances*, 8(50), 28810–28824.
25. Ceccacci, E., & Minucci, S. (2016). Inhibition of histone deacetylases in cancer therapy: Lessons from leukaemia. *British Journal of Cancer*, 114, 605–611.

26. Liang, T., Wang, F., Elhassan, R. M., Cheng, Y., Tang, X., Chen, W., Fang, H., & Hou, X. (2023). Targeting histone deacetylases for cancer therapy: Trends and challenges. *Acta Pharmaceutica Sinica B*, 13(5), 2021–2045.
27. Mustafov, D., Siddiqui, S. S., Kukol, A., Lambrou, G. I., Shagufta, Ahmad, I., & Braoudaki, M. (2024). MicroRNA-dependent mechanisms underlying the function of a β -amino carbonyl compound in glioblastoma cells. *ACS Omega*, 9(29), 31789–31802.
28. Wang, S., Chen, X., Han, X., Hong, X., Li, X., Zhang, H., Li, M., Wang, Z., & Zheng, A. (2023). A review of 3D printing technology in pharmaceuticals: Technology and applications, now and future. *Pharmaceutics*, 15(2), 416.
29. Awad, A., Trenfield, S. J., Gaisford, S., & Basit, A. W. (2018). 3D printed medicines: A new branch of digital healthcare. *International Journal of Pharmaceutics*, 548(1), 586–596.
30. Awad, A., Trenfield, S. J., Goyanes, A., Gaisford, S., & Basit, A. W. (2018). Reshaping drug development using 3D printing. *Drug Discovery Today*, 23(8), 1547–1555.
31. Almaghrabi, M., Alqurshi, A., Jadhav, S. A., Mazzacuva, F., Cilibrizzi, A., Raimi-Abraham, B., & Royall, P. G. (2023). Evaluating thermogravimetric analysis for the measurement of drug loading in mesoporous silica nanoparticles (MSNs). *Thermochimica Acta*, 730, 179616.
32. Vaz, V. M., & Kumar, L. (2021). 3D printing as a promising tool in personalized medicine. *AAPS PharmSciTech*, 22(1), 49.
33. Scherliess, R. (2011). The MTT assay as tool to evaluate and compare excipient toxicity in vitro on respiratory epithelial cells. *International Journal of Pharmaceutics*, 411(1–2), 98–105.
34. Davodabadi, F., Sargazi, S., & Baino, F. (2025). Recent advances in hydrogel based drug delivery systems for enhanced cancer therapy: A review. *International Journal of Biological Macromolecules*.
35. Cervellati, F., Cervellati, C., Romani, A., Cremonini, E., Sticozzi, C., Belmonte, G., Pessina, F., & Valacchi, G. (2014). Hypoxia induces cell damage via oxidative stress in retinal epithelial cells. *Free Radical Research*, 48(3), 303–312.
36. Ledur, P. F., Onzi, G. R., Zong, H., & Lenz, G. (2017). Culture conditions defining glioblastoma cells behavior: What is the impact for novel discoveries? *Oncotarget*, 8(40), 69185–69197.
37. Ashley, E. A. (2016). Towards precision medicine. *Nature Reviews Genetics*, 17(9), 507–522.
38. Dasari, S., & Tchounwou, P. B. (2014). Cisplatin in cancer therapy: Molecular mechanisms of action. *European Journal of Pharmacology*, 740, 364–378.

39. Greene-Schloesser, D., Robbins, M. E., Peiffer, A. M., Shaw, E. G., Wheeler, K. T., & Chan, M. D. (2012). Radiation-induced brain injury: A review. *Frontiers in Oncology*, 2, 73.
40. Javaid, M., & Haleem, A. (2019). Current status and applications of additive manufacturing in dentistry: A literature-based review. *Journal of Oral Biology and Craniofacial Research*, 9(3), 179–185.
41. McFaline-Figueroa, J. R., & Lee, E. Q. (2018). Brain tumors. *The American Journal of Medicine*, 131(8), 874–882. <https://doi.org/10.1016/j.amjmed.2017.12.039>
42. Han, S., Liu, Y., Cai, S. J., Qian, M., Ding, J., Larion, M., Gilbert, M. R., & Yang, C. (2020). IDH mutation in glioma: Molecular mechanisms and potential therapeutic targets. *British Journal of Cancer*, 122(11), 1580–1589.
43. Awad, A., Trenfield, S. J., Gaisford, S., & Basit, A. W. (2018). 3D printed medicines: A new branch of digital healthcare. *International Journal of Pharmaceutics*, 548(1), 586–596. <https://doi.org/10.1016/j.ijpharm.2018.07.024>
44. Awad, A., Trenfield, S. J., Goyanes, A., Gaisford, S., & Basit, A. W. (2018). Reshaping drug development using 3D printing. *Drug Discovery Today*, 23(8), 1547–1555. <https://doi.org/10.1016/j.drudis.2018.05.025>
45. Wang, S., Chen, X., Han, X., Hong, X., Li, X., Zhang, H., Li, M., Wang, Z., & Zheng, A. (2023). A review of 3D printing technology in pharmaceutics: Technology and applications, now and future. *Pharmaceutics*, 15(2), 416.
46. Urbánek, P. and Klotz, L.O. (2017) ‘Posttranscriptional regulation of FOXO expression’, *Journal of Molecular Cell Biology*, 9(3), pp. 200-209
47. Conti, S., Maggi, L., Segale, L., Ochoa Machiste, E., Conte, U., Grenier, P. and Vergnault, G. (2007) Matrices containing NaCMC and HPMC: 1. Dissolution performance characterization’, *International Journal of Pharmaceutics*, 333(1-2), pp. 136-142.
48. Aykul, S. and Martinez-Hackert, E. (2016) Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis’, *Analytical Biochemistry*, 508, pp. 97-103.
49. Neophytou, C. M., Trougakos, I. P., Erin, N., Papageorgis, P., & Papanikolaou, N. (2021). *Apoptosis deregulation and the development of cancer multi-drug resistance. Cancers*, 13(17), 4363.
50. Mattei, V., Santilli, F., Martellucci, S., Chiaramonte, C., Orlando, C., Delle Monache, S. and Colapietro, A. (2021) The importance of tumor stem cells in glioblastoma resistance to therapy’, *International Journal of Molecular Sciences*, 22(8), 3863.
51. Norouzi, M., Nazari, B. and Miller, D.W. (2016) ‘Injectable hydrogel-based drug delivery systems for local cancer therapy’, *Drug Discovery Today*, 21(11), pp. 1835-1849.

52. Franken, N.A.P., Rodermond, H.M., Stap, J., Haveman, J. and van Bree, C. (2006) Clonogenic assay of cells in vitro', *Nature Protocols*, 1(5), pp. 2315-2319.
53. Mavris, M. and Leach, J.K. (2021) Cellular mechanobiology in hydrogel-based synthetic extracellular matrices', *Current Opinion in Biotechnology*, 72, pp. 30-37.
54. Ghasemi, M., Turnbull, T., Sebastian, S. and Kempson, I. (2021) The MTT assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis', *International Journal of Molecular Sciences*, 22(23), 12827.
55. Greijer, A.E. and van der Wall, E. (2004) The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis', *Journal of Clinical Pathology*, 57(10), pp. 1009-1014.
56. Khabibov, M., et al. (2022) Signaling pathways and therapeutic approaches in glioblastoma multiforme', *Signal Transduction and Targeted Therapy*, 7, 105.
57. Wu, H., Liu, Q., Cai, T., Chen, Y.D., Liao, F. and Wang, Z.F. (2022) 'Research Progress on the Regulation Mechanism of Key Signaling Pathways Affecting the Prognosis of Glioma', *Frontiers in Molecular Neuroscience*, 15, 910543.
58. Farhan, M., Silva, M., Xingan, X., Huang, Y. and Zheng, W. (2020) Role of FOXO transcription factors in cancer metabolism and angiogenesis', *Cells*, 9(7), 1586.
59. Wörthmüller, J. and Dobrev, G. (2021) 'MAGI1, a scaffold protein with tumour suppressive and regulatory functions', *Cells*, 10(7), 1694.
60. Paul, S., Xie, S., Yao, X. and Dey, A. (2022) 'Transcriptional regulation of the Hippo pathway', *Cells*, 11(14), 2225.

Appendices

Methods

Scratch (Wound Healing) Assay

U87MG cells were seeded into 12-well plates at a density of 2.5×10^5 cells per well and incubated for 24 hours under standard culture conditions to allow cell attachment and the formation of a confluent monolayer. Following incubation, linear scratches were created as straight lines across each well using a sterile 2 μ L pipette tip to generate a uniform wound area prior to treatment application. The wells were subsequently washed twice with phosphate-buffered saline (PBS) to remove detached cells and cellular debris generated during the scratching process. Treatment conditions were distributed by rows within each plate. The first row was treated with 1% DMSO (negative control). The second row was treated with 200 μ M cisplatin-loaded patches (positive control). The third and fourth rows were treated with SHG-8-loaded patches at concentrations of 40 μ M and 60 μ M. Images of the scratch midlines were captured immediately following treatment application (0 h), and subsequently at 24 h and 48 h post-treatment using a light microscope (Olympus Life Science Solutions, Stansted, UK). Cellular migration and wound closure were analyzed using ImageJ software. The percentage of wound closure at 24 h and 48 h was normalized relative to the initial wound area at 0 h for all treatment groups to facilitate comparative analysis between conditions.

Results

Scratch assay

The scratch wound healing assay was performed to investigate the cell migration of U87MG cells following scratch induction under different treatment conditions. Light microscopy images were obtained at three separate time points, including immediately after scratch formation (0 hours); refer to Figure 10, 24 hours post-scratch; refer to Figure 11, and 48 hours post-scratch; refer to Figure 12 using $\times 40$ magnification. The treatment conditions included DMSO control group, 200 μM cisplatin patch treatment group, 40 μM SHG8 drug patch treatment group, and 60 μM SHG8 drug patch treatment group. For each treatment condition and time point, images were captured from two distinct regions of the scratch area, specifically the top region and the left region of the wound field, in order to provide representative visual documentation of cellular arrangement and scratch morphology across multiple areas of the assay.

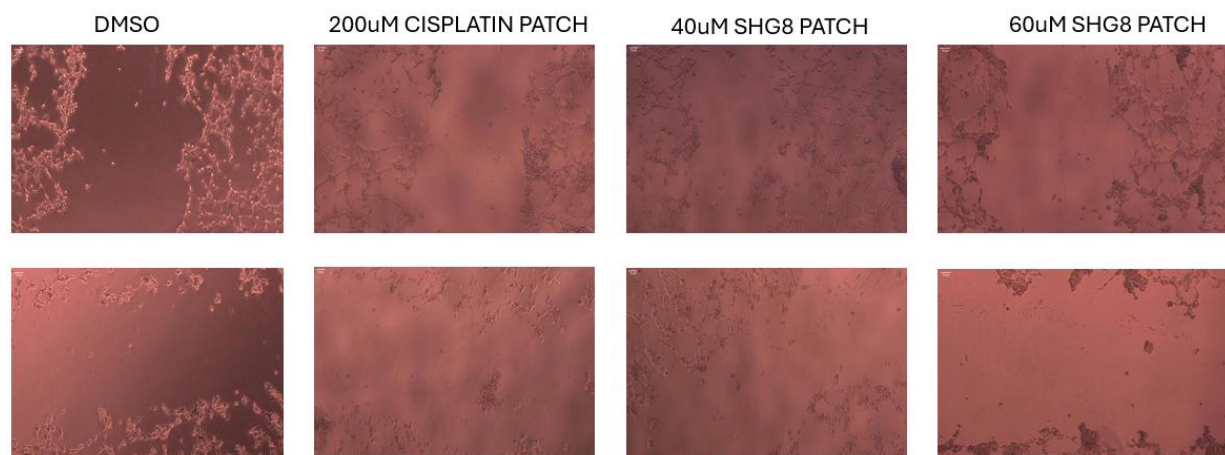


Figure 10: U87MG cells images taken 0 hours after scratch. Figure 10 presents representative brightfield microscopy images of U87MG cells immediately following scratch induction (0 hours) at $\times 40$ magnification under different treatment conditions. The experimental groups shown include the DMSO control, a 200 μM cisplatin patch treatment, a 40 μM SHG8 drug patch treatment, and a 60 μM SHG8 drug patch treatment. For each treatment condition, two regions of the scratch area were captured and presented. Images shown in the first row correspond to the top region of the scratch, while images shown in the second row correspond to the left region of the scratch.

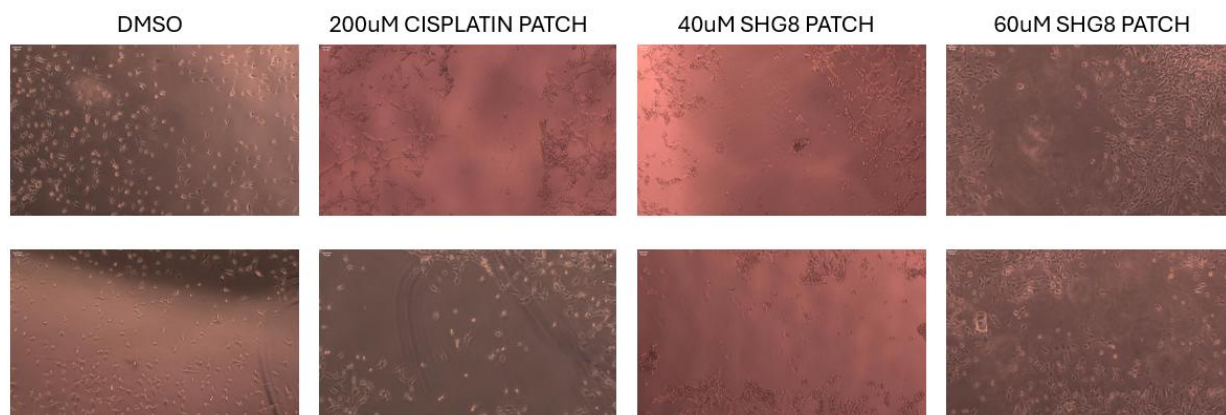


Figure 11: U87MG cells images taken 24 hours after scratch. Figure 11 presents representative brightfield microscopy images of U87MG cells captured 24 hours after scratch induction at $\times 40$ magnification under different treatment conditions. The experimental groups shown include the DMSO control, a 200 μM cisplatin patch treatment, a 40 μM SHG8 drug patch treatment, and a 60 μM SHG8 drug patch treatment. For each treatment condition, two regions of the scratch area were captured and presented. Images shown in the first row correspond to the top region of the scratch, while images shown in the second row correspond to the left region of the scratch.

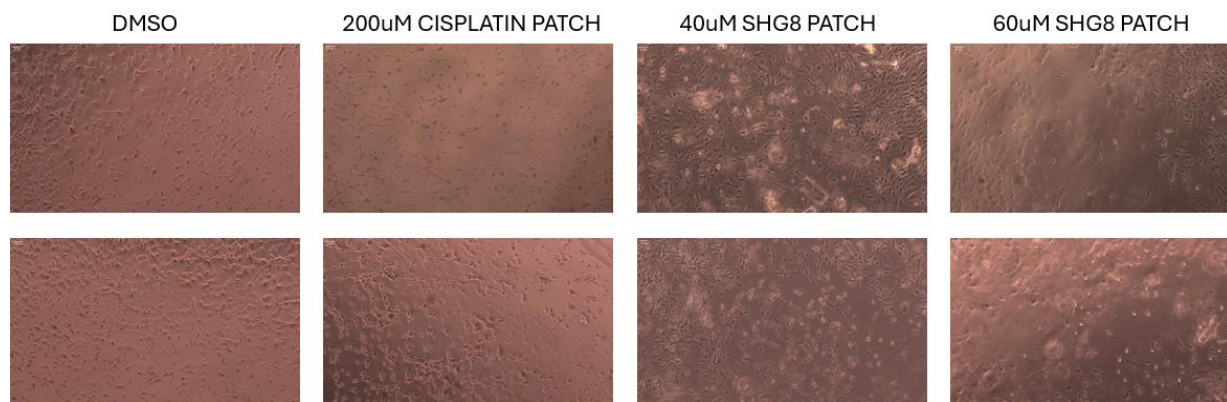


Figure 12: U87MG cells taken 48 hours after scratch. Figure 12 presents representative brightfield microscopy images of U87MG cells captured 48 hours after scratch induction at $\times 40$ magnification under different treatment conditions. The experimental groups shown include the DMSO control, a 200 μM cisplatin patch treatment, a 40 μM SHG8 drug patch treatment, and a 60 μM SHG8 drug patch treatment. For each treatment condition, two regions of the scratch area were captured and presented. Images shown in the first row correspond to the top region of the scratch, while images shown in the second row correspond to the left region of the scratch.

A wound healing scratch assay was conducted to assess the effect of SHG-8 drug patch treatment on wound healing area over time in U87MG cells. Cells were treated with DMSO, 200 μ M cisplatin, and SHG-8 drug patch concentrations of 40 μ M and 60 μ M, and wound healing area measurements were recorded at 0, 24, and 48 hours following treatment. Data obtained from the assay were used to compare wound healing area changes across the different treatment groups over the experimental time period.

Effect of SHG-8 Drug Patch on Wound Healing Area over time

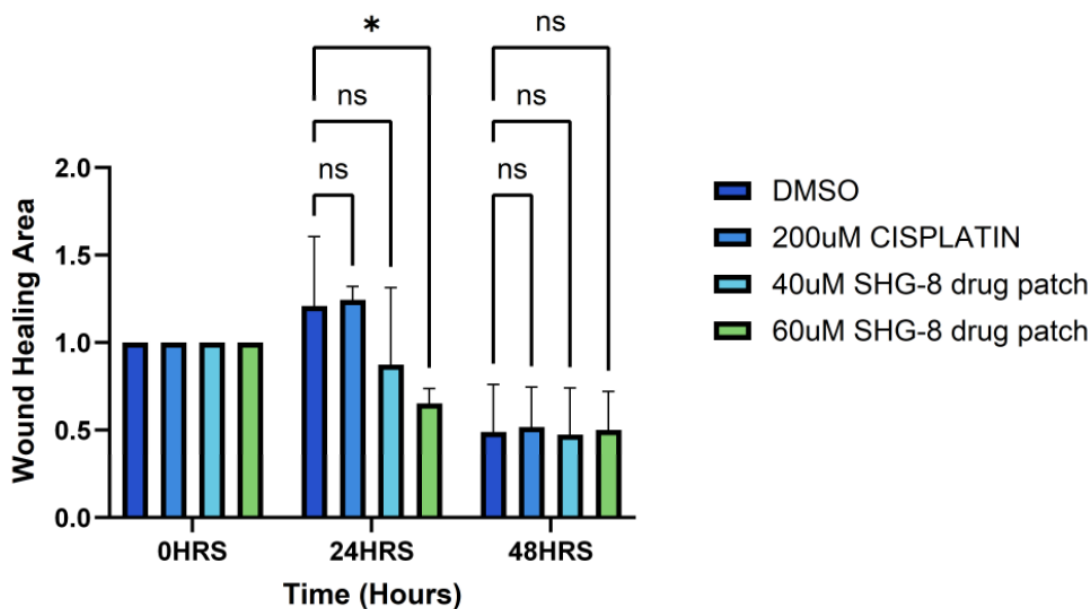


Figure 13: Effect of SHG-8 Drug Patch on Wound Healing Area Over Time. Scratch assay analysis was performed to evaluate the effect of SHG-8 drug patch treatment on wound healing area in comparison with DMSO and 200 μ M cisplatin controls over 48 hours. Wound healing area was measured at 0, 24, and 48 hours following treatment. Data are presented as mean $\pm$ standard deviation (SD) from three independent replicates. Statistical analysis showed a significant reduction in wound healing area at 24 hours in the 60 μ M SHG-8 drug patch treatment group compared with control ($p < 0.05$), while other comparisons were not statistically significant (ns).

