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Development Of A Novel Preclinical Medulloblastoma Model Through Stable Diphtheria Toxin Receptor Expression And Diphtheria Toxin-Mediated Ablation

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Abstract

Despite advances in therapeutic approaches, cancer represents a major global health problem, with increasing incidence worldwide. Improvements in treatment approaches have simultaneously increased survival rates, resulting in a growing population of cancer survivors. Survivorship has gained prominence as an essential research area, as many patients, particularly the paediatric population with medulloblastomas, experience long-term cognitive, neurological, and emotional effects that persist beyond the scope of initial treatment and substantially affect quality of life. These challenges highlight the need for the development of effective strategies that enable selective manipulation of tumour cell populations within a physiologically relevant model to better understand and mitigate long-term treatment-related side effects. This study, therefore, aimed to address this gap by developing a targeted controllable ablation platform.

This study aimed to genetically engineer Sonic Hedgehog medulloblastoma cell lines to express Diphtheria Toxin Receptors (DTR), utilising the DTR/DT system, thereby enabling receptor-mediated, ligand-specific ablation of targeted cells following exposure to Diphtheria Toxin (DT). Two well-characterised, patient-derived medulloblastoma cell lines, ONS-76 and DAOY, were selected to provide a physiologically relevant model. Cells were cultured and transduced with a lentiviral vector encoding the DTR and GFP reporter. The cells were then selected for stable clones using puromycin, and successful DTR expression was confirmed by GFP Fluorescence and FACS analysis. Cytotoxic responses to DT were assessed in both 2D monolayer and 3D spheroid cultures using PrestoBlue and CellTiter-Glo assays, respectively, to characterise dose-dependent and cell-line-specific responses.

Stable Diphtheria Toxin Receptor expression was achieved in ONS-76 and DAOY cells, validated by GFP fluorescence. Optimised 3D spheroids provided a more physiologically relevant model. Diphtheria toxin cytotoxicity was selectively
observed on ONS-76-DTR and DAOY-DTR spheroids, with DAOY-DTR spheroids requiring higher concentrations to elicit a comparable response. These findings demonstrate cell line-specific responses and highlight this model's use for targeted ablation studies.

The DTR/DT model offers a robust, selective, and physiologically relevant platform for generating a targeted ablation model of medulloblastoma in ONS-76 and DAOY cell lines. Its significance lies in its ability to study long-term survivorship effects, supporting the development of neuroprotective therapeutic advances, and offering a tool to improve the quality of life for paediatric cancer survivors.

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1.0 Introduction

Cancer remains a major global health and economic burden, with lifetime incidence estimated at 44.85% for men and 38.08% for women (Dorak & Karpuzoglu, 2012). According to the World Health Organisation (WHO, 2023), it was the second leading cause of death worldwide, with over 8.97 million cancer-related deaths reported in 2023. These figures are based on data from the 2016 GLOBOCAN study, which continues to inform global cancer surveillance. Projections indicate a rapid rise in incidence, with an estimated 29.9 million new cases and 15.3 million deaths expected by 2024. These statistics highlight the substantial and growing global burden of cancer, justifying the extensive urgency for research efforts devoted to developing improved diagnostic and therapeutic interventions.

The most frequently diagnosed cancer types include lung (2.5 million cases annually), female breast (2.3 million), colorectal (1.9 million), and prostate cancer (1.5 million) (WHO, 2024). Despite advances in early detection and treatment, these numbers highlight the immense and ongoing pressure cancer places on healthcare systems. Beyond diagnosis and treatment, the period following active treatment, referred to as survivorship, has become an increasingly important focus of research. Survivors often face lasting physical, cognitive, and emotional effects, and evidence shows that cancer and its treatment can impair quality of life (QoL) long after remission.

Cancer survivorship is a relatively new focus in clinical research, yet evidence suggests that the disease and its treatment can significantly impair QoL long after remission. Within this broader context, childhood cancers present unique challenges. Survivors may live for decades after treatment but frequently experience persistent neurological, cognitive, and psychosocial difficulties. Medulloblastoma, the most common malignant brain tumour in children, exemplifies this challenge. While survival rates have improved with surgery, radiotherapy, and chemotherapy, these aggressive interventions often result in long-term neurological consequences (Dietrich, 2010; Kumar et al., 2025).

This thesis integrates a critical review of the existing literature on the long-term neurological effects of medulloblastoma in childhood cancer survivors, while developing experimental tools to assess these outcomes. By focusing on objective neurological endpoints, it seeks to enhance understanding of the lasting effects of treatment and to inform future rehabilitation and quality-of-life strategies.

1.1 Cancer Incidence and Survival Rates

Trends in cancer incidence have fluctuated over time due to a combination of medical advancements, evolving societal attitudes toward screening, and changes in lifestyle and environmental exposures. Lifestyle and environmental factors play a substantial role in cancer development, with behaviours such as alcohol consumption, poor diet, physical inactivity, smoking, and exposure to environmental pollutants contributing significantly to overall risk (MacMillan,2023). Smoking remains the leading cause of lung

cancer, with approximately 90% of cases occurring among current or former smokers, particularly those who began smoking at an early age (MacMillan, 2023).

Melanoma, a form of skin cancer, is strongly linked to artificial tanning and excessive sun exposure, with incidence rates rising by 147% since 1994 (Cancer Research UK, 2019). The use of sunbeds increases the risk of skin cancer by 20%, and this rises to 87% when use begins before the age of 35 (Knight, 2012).

The global incidence of kidney cancer has also increased among both men and women, with higher mortality rates observed in individuals from lower socioeconomic backgrounds (Cirillo et al., 2024). While the exact causes of this rise remain unclear, the American Cancer Society (2024) suggests that contributing factors may include an ageing population and occupational exposure to environmental toxins. In contrast, the incidence of stomach cancer in women is declining, possibly due to improved food preservation practices and a reduction in Helicobacter pylori infections (Ilic & Ilic, 2022). In addition to societal influences, biological factors such as age and genetics significantly affect cancer incidence rates. Age is a key determinant, with incidence increasing notably as individuals reach mid-life (White et al., 2014). Foster et al. (2014) attribute this rise to age-related declines in immune function, notably reduced T-cell proliferation, which leaves the body more susceptible to malignancies due to weakened immune surveillance (Fu et al., 2023).

Genetic predisposition is another crucial factor that influences cancer risk (Zhu et al., 2021). Mutations in genes responsible for regulating the cell cycle and repairing DNA can increase susceptibility to specific types of cancer. For instance, mutations in BRCA1, BRCA2, and PALB2, genes involved in DNA repair, can significantly increase the risk of breast, ovarian, prostate, and pancreatic cancers (Kuchenbaecker et al., 2017; Nyberg et al., 2020). Notably, up to 50% of women with a PALB2 mutation may develop breast cancer by age 70 (Tanday, 2014). These genetic variations differ across populations, contributing to global disparities in cancer incidence.

A range of clinical and demographic variables, including tumour location, disease stage at diagnosis, patient age, and biological sex, influences cancer survival rates. Understanding these variables is essential for improving prognostic models and reducing mortality (Halabi & Owzar, 2010). The overall five-year survival rate for all cancers is approximately 62.7% (Gloeckler Ries, 2003). However, this aggregated figure oversimplifies the variation between cancer types and demographic groups. Tumour location is among the most critical determinants of survival. In brain cancers, prognosis can differ markedly depending on the region affected. Tumours in the brainstem or cerebellum generally have poorer outcomes than those in the cerebral lobes, due to the vital functions controlled by these regions and the complexity of surgical intervention (Hossain et al., 2021). Such disparities underscore the need for location-specific strategies in cancer treatment and highlight the inherent heterogeneity of cancer subtypes (Krzyszczyk et al., 2018).

The stage of tumour detection also plays a pivotal role in survival outcomes. Early-stage diagnoses (Stages 1 and 2) are associated with significantly better prognoses compared

with advanced stages (Stages 3 and 4) (McPhail, 2016). For example, Stage 1 melanoma has a five-year survival rate exceeding 90%, which drops to roughly 30% at Stage 4 (Heistein et al., 2024). This gap reflects the impact of metastasis and reinforces the importance of early detection, public awareness campaigns, expanded screening, and improved healthcare access (Crosby et al., 2022).

1.2 Cancer Treatment Modalities

Cancer is primarily treated through three primary modalities: surgery, chemotherapy, and radiation therapy, which may be used individually or in combination. More recently, advances in hormone therapy, immunotherapy, and immunotoxin-based treatments have expanded therapeutic options (Charmsaz et al., 2019). Surgery aims to reduce tumour burden or remove malignant tissue entirely (National Cancer Institute, 2015). Surgical approaches include palliative surgery (symptom relief), prophylactic surgery (risk reduction in high-risk individuals), and curative surgery (complete tumour removal). Suitability depends on tumour location, size, and patient factors such as age. For example, paediatric patients may require modified approaches (Pinkerton et al., 1996). In children, surgery is often complicated by the need to preserve growth potential and neurological function. Neurosurgical procedures for paediatric brain tumours, for example, carry higher risks of long-term cognitive or endocrine deficits compared to adults (Trapani & Murdaugh, 2022).

Post-surgical side effects can be substantial. Persistent or chronic pain is common (Habibi et al., 2022), varying in severity with the extent of surgery and tumour location (Grisham, 2024). Research suggests that surgery and anaesthesia may also influence tumour metastasis risk via inflammatory and immune-modulatory pathways (Cole et al.,2015; Ramirez et al., 2023). These effects include sympathetic activation, altered expression of cancer genes, increased angiogenesis, and suppression of cell-mediated immunity (Angele & Faist, 2002; Kim, 2018). Opioids used for post-operative pain may further contribute to immunosuppression and promote tumour growth in cancers expressing μ-opioid receptors (MOR) (Yu et al., 2022; Amaram-Davila et al., 2020).

Radiation therapy utilises high-energy particles (electrons, protons, and neutrons) or electromagnetic waves (X-rays) to damage the DNA of cancer cells, thereby preventing their replication (Mayo Clinic, 2020; National Cancer Institute, 2019). External beam radiation is the most common form, precisely targeting tumours while sparing healthy tissue (Maani & Maani, 2022). Internal radiation (brachytherapy) delivers radioactive material directly to the tumour, such as via the hepatic vasculature in liver cancer (Pollock et al., 2022). Paediatric patients are especially vulnerable to radiation-induced injury in the developing brain and spine, leading to long-term effects such as impaired learning, memory difficulties, endocrine dysfunction, and reduced growth. To limit these effects, techniques such as proton beam therapy and careful dose modulation are increasingly used in children (Thomas & Timmermann, 2020). Side effects include fatigue, nausea, mucositis, and skin damage, with radiation-induced skin reaction (RISR) being widespread (Chan et al., 2014; Glover & Harmer, 2014). RISR may require interventions such as steroid creams or hydrogel dressings (Burke et al., 2021) and, if severe, can interrupt treatment, increasing recurrence risk.

Chemotherapy employs cytotoxic drugs to destroy cancer cells and prevent metastasis (Hossain & Hossain, 2023). Common classes of chemotherapy agents include antimetabolites, alkylating agents, and anthracyclines, such as doxorubicin, which disrupt DNA synthesis and cause strand breakage through intercalation and the formation of free radicals (Dorr & Von Hoff, 2015). Side effects range from fatigue and nausea to chemotherapy-induced peripheral neuropathy (CIPN), which affects up to 60% of patients (Zhang, 2021). CIPN results from nerve damage and can cause persistent pain, tingling or numbness (Wolf et al., 2012; Banach et al., 2016). While symptom management strategies exist, such as antidepressants, anticonvulsants, and physical therapies (Colvin,2019), no definitive treatment for CIPN is yet available (Ma et al., 2018). In children, chemotherapy is a mainstay of treatment. However, certain drugs such as cisplatin and methotrexate are linked to specific long-term toxicities, including hearing loss (Callejo et al., 2015), fertility impairment (Sawhney et al., 2005) and delayed neurocognitive development (Ellen et al., 2015). These risks make survivorship care and follow-up critical in paediatric oncology to improve the QoL for cancer survivors.

Combination therapy often enhances efficacy, with surgery supported by chemotherapy or radiation to reduce the risk of recurrence (Diaz-Nieto et al., 2013; Mayo Clinic, 2020a). While this multimodal approach can improve five-year survival rates by more than 25% (Sun Ha Jee et al., 2011), it also increases the cumulative burden of side effects, impacting long-term QoL (Haidinger & Bauerfeind, 2019).

In paediatric oncology, multimodal regimens are often risk-adapted, balancing treatment intensity with the need to preserve long-term growth, development, and QoL. The heightened vulnerability of the developing brain means that children are at particular risk of long-term developmental consequences, including cognitive, endocrine, and psychosocial impairments (Weiss, 2000; De Ruiter et al., 2012; Mulhern et al., 2004). To reduce these risks, children with lower-risk cancers may receive reduced doses of radiation or chemotherapy, while higher-risk groups may undergo intensified protocols.

While advances in treatment strategies have significantly improved five-year survival rates, these gains are often accompanied by considerable challenges to long-term survivorship (Girardi et al., 2019). As a result, current research is increasingly highlighting the importance of neuroprotective interventions to reduce the enduring effects of treatment. In light of the profound neurological and quality-of-life consequences associated with cancer therapies, this study concentrates on childhood cancers of the central nervous system (CNS), with a particular focus on medulloblastoma and its lasting impact on survivors.

1.3 Medulloblastoma Incidence Rates

The National Cancer Institute (2011) defines medulloblastoma as a fast-growing malignant tumour originating in the cerebellum, a region essential for coordinating locomotion and balance (Morton & Bastian, 2004). Although the precise mechanisms

of tumour initiation remain unclear, evidence suggests it arises from granule cell precursors in the external germinal layer of the developing cerebellum (Mahapatra & Amsbaugh, 2023). This region plays a crucial role in early brain development and the formation of neural networks (Kim et al., 2023). Dysregulation of granule cell precursor function, particularly their involvement in signalling pathways such as the Sonic Hedgehog (Shh) pathway, has been implicated in medulloblastoma progression (Polkinghorn & Tarbell, 2007; Srivastava & Nalbantoglu, 2010; Tamayo-Orrego & Charron, 2019). Between 1954 and 1997, the incidence of medulloblastoma in the UK was approximately 4.5 cases per million children (Alston et al.,2007). In adults, however, medulloblastoma is a rare occurrence, accounting for only 0.4-1% of all primary brain tumours (Bartlett et al., 2013), reflecting its strong predilection for the paediatric population. While most cases show a slight male predominance, this pattern varies across molecular subgroups, including the Wingless (Wnt), Sonic Hedgehog (SHH), Group 3 and Group 4 subtypes, which are defined by distinct genetic features. The majority of diagnoses occur between the ages of 5 and 7 (Roberts et al., 1991), underscoring the importance of early detection and timely intervention.

1.4 The Role of the Cerebellum in Medulloblastomas

Medulloblastomas predominantly occur in the midline of the cerebellum (Tae Sung Park et al., 1983) and are prone to local spread to adjacent regions, such as the brainstem and fourth ventricle (Mahapatra & Amsbaugh, 2023; Tae Sung Park et al., 1983), complicating the treatment of these tumours. Additionally, due to their proximity to various brain structures, medulloblastomas can disseminate to other parts of the body via the cerebrospinal fluid circulating through the central nervous system (Mahapatra & Amsbaugh, 2023), potentially leading to secondary tumours (Garzia et al., 2018). The cerebellar location of these tumours can disrupt normal cerebellar functions. Under typical conditions, the cerebellum coordinates movement and balance, which means medulloblastomas can result in physical symptoms such as clumsiness and loss of coordination. Moreover, raised intracranial pressure due to tumour growth can obstruct cerebrospinal fluid flow, leading to headaches, nausea, blurred vision, and lethargy (Raywat Noiphithak et al., 2021).

The cerebellar cortex, which is rich in neurons, comprises approximately 80% of the brain's neurons (Roostaei et al., 2014). It comprises granule and Purkinje cells, which are essential for coordinating excitatory and afferent inputs, as well as motor outputs, from the cerebellum. Structurally, the cerebellum is composed of three distinct layers: the molecular layer, the Purkinje cell layer, and the granular layer (Unverdi & Alsayouri, 2021). Granule cells, the most populous within the cerebellum (Consalez et al., 2021), receive input from mossy fibres and then transmit this to Purkinje cells, enabling the differentiation between varying input patterns (Kim et al., 2023). Purkinje cells play a crucial role in coordination and motor learning, as they integrate information from various sources to transmit inhibitory and modulatory signals to deep areas of the cerebellum, thereby modulating movement (Tabata & Kano, 2006).

The cerebellum plays a role in autonomic functions (Berman et al., 1974) and contributes to cognitive and emotional processes. Recent studies by De Zeeuw and Yeo (2005) suggest that the cerebellum is involved in memory formation through efferent and afferent connections, as shown in Figure 2. It integrates data from voluntary muscle movements to coordinate responses and maintain balance, receiving input from the vestibular nuclei (Jimsheleishvili & Dididze, 2023). This has led to the hypothesis that the cerebellum continuously learns to predict the precise timing of multiple stimuli, thereby triggering accurate responses (D'Angelo, 2018).

Li, Keay, and colleagues (2024) suggest that the cerebellum plays a modulatory role in pain processing through its connections with higher-order sensorimotor, cognitive, and emotional centres. Considerable evidence from neuroimaging further supports this view, showing cerebellar activation in response to painful stimuli in both acute and chronic pain states (Moulton et al., 2010). During chronic pain, persistent activation of cerebellar regions such as Crus I, lobules VI, and VIII has been observed, linking the cerebellum to both the affective and sensory dimensions of pain (Manda et al., 2025). In addition, increased blood flow to specific cerebellar regions, including the vermis, has been reported in individuals with chronic pain, underscoring the cerebellum's role across both acute and chronic pain processing (Li, Keay, et al., 2024).

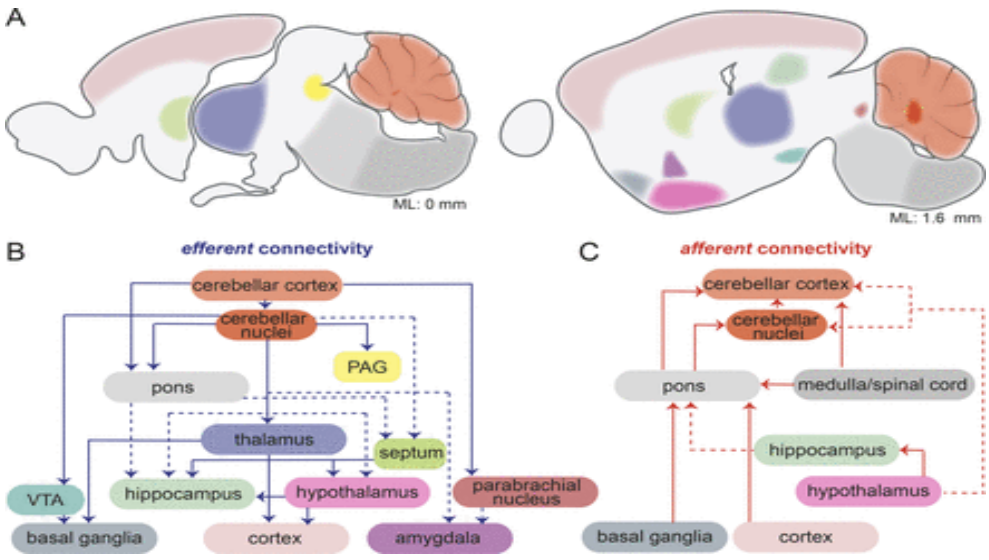


Figure 1A depicts sagittal sections of the rat brain, with corresponding colour-coded brain regions mapped to connectivity in B and C. The connectivity maps in B and C illustrate the efferent (B) and afferent (C) pathways linking the cerebellum. These connections facilitate both cognitive and emotional processing. Pathways connecting the cerebellum, prefrontal cortex, hippocampus, and thalamus primarily play a role in cognitive functions, whereas circuits linking the amygdala, hypothalamus, and ventral tegmental area mainly contribute to emotional and affective processing. Solid lines represent direct connections, whereas dotted lines indicate indirect or not yet elucidated connections. The image was obtained from a paper by Rudolph et al. (2023).

Cerebellar dysfunction has been linked to several neurocognitive disorders, including attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (Rudolph et al., 2023). A growing body of research indicates that the cerebellum plays a crucial role in the emotional processing of fear and anxiety (Frontera et al., 2023; Moreno-Rius,

2018; Zhang et al., 2024). The cerebellum's role in modulating both emotional and sensory information also extends to pain perception and response (Moreno-Rius, 2018). Experimental evidence suggests that electrical and chemical stimulation of the cerebellum can alter nociceptive responses, whereas cerebellar damage has been associated with increased pain sensitivity and reduced analgesia (Ruscheweyh et al., 2014; Saab et al., 2001).

Furthermore, the affective dimension of pain is thought to be mediated by the cerebellum's extensive connections with limbic structures such as the amygdala, hippocampus, and prefrontal cortex, regions central to emotional and cognitive processing (Schmahmann, 2019).

Longitudinal studies assessing chemotherapy-induced changes have provided critical insights into the mechanisms behind cognitive dysfunction. Evidence from a study by McDonald et al. (2010) has shown that patients post-chemotherapy exhibit significant reductions in grey matter volume, as well as in cerebral blood flow in key brain regions such as the frontal cortex and cerebellum (Li & Caeyenberghs, 2018). Emerging research suggests that changes in brain regions are primarily attributable to the method of treatment itself, rather than the presence of the tumour, thereby contributing to the observed cognitive impairments (Országhová et al., 2021).

1.5 WHO Categorisation of Medulloblastoma Subtype Categorisation and Distinct Features

Medulloblastomas are categorised into four distinct molecular groups: Wingless-related Integration Site (Wnt), Sonic Hedgehog (Shh), Group 3, and Group 4 (Taylor et al., 2012). The Wnt and Shh subtypes are named after the signalling pathways they activate, and these groups have garnered considerable attention in therapeutic research due to their unique molecular profiles and varying clinical outcomes. On the other hand, the primary signalling pathways associated with Groups 3 and 4 remain largely undefined, which is why these groups have been assigned more generic names (Taylor et al., 2012). While specific subgroups within these four categories have been proposed, the characterisation of each subgroup has yet to be established, resulting in the absence of an official classification system.

Among these, the Wnt-pathway medulloblastoma presents the highest survival rate, with upwards of 90% of patients surviving beyond five years (Mani et al., 2023). For those who do not survive, the causes are often linked to complications arising from treatment or the development of secondary neoplasms rather than the recurrence of Wnt growth (Ellison et al., 2011). Tumours classified under Wnt medulloblastoma typically exhibit CTNNB1 mutations (Liu et al., 2014) and the deletion of one copy of chromosome 6 within the tumour (Taylor et al., 2012). Additionally, there is a notable overexpression of genes associated with Wnt signalling pathways, a finding that has also been observed in the Shh and Group 3 categories (Northcott et al., 2011).

The Shh-pathway medulloblastoma, which is characterised by its marked overactivation and dysregulation of the Shh signalling pathway (Smit et al., 2022), accounts for approximately 30% of all medulloblastoma cases (Tamayo-Orrego & Charron, 2019). The mechanisms underlying this pathway are influenced by patient age; specific mutations, such as the SUFU mutation, are predominantly found in infant patients, whereas mutations such as GL12, NMYC, SMO, and TP53 are more frequently identified in older patients (Smit et al., 2022). This age-dependent variation in mutation prevalence highlights the complexity of Shh pathway dysregulation and its implications for treatment strategies.

The Group 3 subcategory of medulloblastomas is referred to as 'classic', comprising predominantly large cell/anaplastic (LCA) tumours. In addition, due to the amplification of MYC expression compared to Grop4 and Wnt subgroups, it has been suggested that Group 3 tumours be renamed the MYC group (Hatten & Roussel, 2011). Further overexpression of the oncogene OTX2 has been observed exclusively in Group 3 and 4 tumours (Kleinschmidt-DeMasters et al., 2006; Northcott et al., 2019). Group 3 tumours are more prevalent in infants and children than adults, with a higher occurrence in males (Taylor et al., 2012). Group 3 has the worst prognosis rates compared to other subgroups, with a five-year survival rate of 50%, which is lower than that of the categorised subgroups (Thompson et al., 2017). This poor prognosis is partly due to increased angiogenesis, a process that promotes the growth of new blood vessels, which is driven by VEGF overexpression (Shanmugham et al., 2004).

Group 4 medulloblastomas account for approximately 34–40% of all cases (Kool et al., 2012) and are twice as common in males as in females. This sex difference has been attributed to the loss of an X chromosome in around 80% of female Group 4 cases (Taylor et al., 2012). Although specific molecular markers for Group 4 remain unclear, KCNA1, a potassium channel, is widely used as an immunohistochemical marker for tumour identification (Cho et al., 2011; Kijima & Kanemura, 2016). Prognosis varies according to risk stratification: patients with standard-risk Group 4 tumours show a five-year survival rate of approximately 80%, compared with 60% for those with high-risk tumours.”

Understanding the molecular intricacies of medulloblastomas, particularly the Wnt and Shh subtypes, is crucial for developing targeted therapies and improving patient clinical outcomes. Continued research is essential to elucidate further the signalling pathways associated with Groups 3 and 4 and to refine the classification of these tumours, thereby enhancing therapeutic approaches and improving patient outcomes.

1.6 Histological Categorisation of Medulloblastomas

Medulloblastomas can be histologically classified into several distinct variants, including classic, desmoplastic/nodular, anaplastic, and large cell types, each possessing unique characteristics that are critical for accurate diagnosis and treatment (Raabe & Eberhart,

2010). Among these variants, the classic type is the most prevalent, accounting for approximately 72% of all medulloblastoma cases (Louis et al., 2016). The diagnostic criteria for classic medulloblastomas are well-established and include distinct histological features.

Classic medulloblastomas are characterised by relatively small, circular nuclei, notably smaller than red blood cells. Additionally, these tumours are distinguished by the absence of detectable mitotic activity, which is a key indicator of their cellular nature (Orr, 2020). A clear understanding of these classifications and characteristics is crucial for medical professionals to develop effective treatment strategies and improve patient outcomes (Damato et al., 2009).

Desmoplastic/nodular medulloblastomas (DNMB) represent distinct subtypes with unique characteristics. These tumours are predominantly identified by their formation of nodules, which showcase neuroglial differentiation. Surrounding these nodules is a dense deposition of pericellular collagen, providing a distinctive histological feature that can be observed under microscopic examination. This characteristic collagenous environment further influences the tumour's behaviour and interaction with surrounding tissues, playing a crucial role in tumour biology (Orr, 2020).

The occurrence of DNMB is predominantly observed in the paediatric demographic, making it a critical focus for researchers and clinicians working in paediatric oncology. Extensive research efforts to understand this subtype of medulloblastoma have substantially reduced mortality rates and improved patient outcomes. The significant global emphasis on DNMB, driven by various research groups and clinical trials, has markedly progressed the development of targeted therapies and optimised treatment options for affected children (AbdelBaki et al., 2017).

A pivotal molecular characteristic of DNMB is its ability to activate the Sonic Hedgehog (Shh) signalling pathway. The activation of this pathway plays a crucial role in the tumorigenesis of DNMB, leading to the classification of these tumours as Shh-activated medulloblastoma subgroups. This classification not only aids in understanding the underlying biological mechanisms but also has important implications for treatment strategies. It emphasises the necessity for tailored therapeutic approaches that address the specific molecular profile of these tumours, highlighting the importance of personalised medicine in oncology (Taylor et al., 2012).

1.7 Improvement of Cancer Survival Rates

Over the past 50 years, cancer survival rates in the UK have improved substantially, with overall rates roughly doubling during this period (Cancer Research UK, 2015). These gains are largely attributed to advances in diagnostic methods and the development of more effective therapies. Cancers detected earlier, such as breast cancer, generally demonstrate higher five-year survival rates, whereas those often diagnosed at advanced stages, such as lung cancer, have markedly poorer outcomes (City of Hope, 2023). Survival also varies with age, typically declining in older populations (Cancer Research UK, 2015), which underscores the unique characteristics of paediatric cancer.

Childhood cancers differ biologically from adult cancers in their causes, treatment strategies, side effects, clinical presentation, and survival outcomes (Kerchner, 2024). Paediatric cancers account for less than 1% of all diagnoses, with the highest incidence occurring in children under five years of age (Cancer Research UK, 2015d). Advances in medical treatment have substantially increased five-year survival rates for childhood cancers, now reaching approximately 80% for most types (Office for National Statistics, 2018). However, survival differs considerably by cancer subtype: acute lymphoblastic leukaemia (ALL) has a five-year survival rate exceeding 90% (Vora et al., 2013), whereas glioblastomas remain associated with poor outcomes, with fewer than 40% of patients surviving beyond five years (Singla et al., 2021).

1.8 Risk Factors for Childhood Cancer Subtype

In contrast to adult cancers, childhood cancers are less frequently linked to environmental or lifestyle factors and are predominantly driven by genetic mutations or developmental abnormalities (Slavin & Wiesner, 2009; National Cancer Institute, 2017). Mutations in cancer-predisposition genes account for at least 10% of paediatric cancer cases (Brodeur et al., 2017), and certain inherited genetic conditions substantially elevate risk. For example, children with Down syndrome are markedly more likely to develop acute leukaemia (Baruchel et al., 2023), while individuals with Li-Fraumeni syndrome face an increased risk of multiple cancers, including breast and brain tumours (Schneider et al., 2019). Paediatric cancers may also arise from spontaneous genetic mutations during early development, although the underlying mechanisms remain incompletely understood (Moore et al., 2021).

The distribution of cancer types in paediatric populations differs significantly from that in adults. Leukaemia, brain tumours, and lymphomas are the most common childhood cancers (National Cancer Institute, 2019). Acute lymphoblastic leukaemia (ALL) alone accounts for approximately 30% of all cases (Namayandeh et al., 2020). Brain tumours, including medulloblastomas and gliomas, account for approximately 25% of diagnoses, with the highest incidence reported in the United States (Subramanian & Ahmad, 2020). These differences in distribution highlight the unique biological and developmental characteristics of childhood cancers compared to adult malignancies.

Survival rates also vary widely across regions. In high-income countries, advances in treatment have led to substantial improvements, whereas in many low-income settings, survival remains below 30% (Farrag et al., 2023). These disparities underscore the ongoing challenges in achieving equitable access to high-quality care, a critical factor not only for improving survival but also for mitigating the long-term consequences of treatment. Understanding the genetic and developmental risk factors specific to childhood cancers is therefore essential for informing early detection, targeted therapies, and strategies to enhance survivorship outcomes and treatment.

1.9 Long-Term Effects of Early Life: Pain in Paediatric Cancer Survivors

Although survival rates for childhood cancer have improved markedly, survivors often experience a range of long-term side effects resulting from treatment, the tumour itself, or the combined psychological impact on the individual and their family. Treatments such as chemotherapy, radiation, and surgery, while effective at eradicating cancer cells, can cause enduring complications (Gegechkori et al., 2017). These long-term health challenges frequently develop gradually and may worsen over time (Lustberg et al., 2023). Chemotherapy, in particular, can damage healthy tissues surrounding tumours, contributing to persistent physical and psychological effects, including fatigue, neuropathy, and mental health issues (Yarana & St. Clair, 2017; Gegechkori et al., 2017). Survivors also face an elevated risk of secondary cancers or neoplasms, with approximately 30% of secondary malignancies occurring within five years of the initial diagnosis (Pourhassan et al., 2024; Youlden et al., 2019).

1.10 Quality of Life, Long Lasting Pain and Cognitive Outcomes

Cancer and its treatments have far-reaching effects that extend beyond tumour elimination, significantly impacting survivors' quality of life (QoL) across multiple domains. Survivors frequently experience persistent emotional, cognitive, and physical challenges that continue into adulthood, resulting in substantial reductions in QoL (Board et al., 2003). Chronic pain, in particular, is a common and enduring complication, affecting over half of survivors, and contributing to considerable cognitive and psychological distress. These challenges compromise daily functioning and overall well-being (Tonning Olsson et al., 2020).

The cumulative impact on survivors' QoL is profound, influencing physical health, mental health, and social functioning, underscoring the need for strategies that mitigate long-term treatment effects (Yuzhalin, 2024). Some survivors never fully recover from the cognitive and emotional effects of cancer treatment, leaving lasting deficits (Wefel et al., 2004). While the mechanisms underlying these deficits remain incompletely understood, evidence suggests they are often linked to hippocampal damage caused by chemotherapy (Crandall et al., 2004; Winocur et al., 2006). Such damage can lead to impairments in processing speed (Chevignard et al., 2016), memory (De Ruiter et al., 2012), and executive function (Edelstein et al., 2011), which typically become more pronounced as survivors enter early adulthood (Moxon-Emre et al., 2016).

Early intervention is critical for mitigating these cognitive challenges. Tailored therapeutic strategies can be implemented to address both cognitive and emotional effects of treatment, thereby enhancing QoL and supporting better overall functioning in daily life (Von Ah et al., 2021).

1.11 Implications of Early Life Pain

Early life pain experiences (such as those related to cancer) cause changes in long-term stress reactivity, leading to heightened heart rate and cortisol levels well into adulthood (Ranger & Grunau, 2014). Emerging research shows that this is mediated by stress changing the functionality of the hypothalamic-pituitary-adrenal (HPA) axis, leading to maladaptive responses to anxiety and stress-provoking stimuli (Brummelte et al., 2015), hence leading to a 45% increase in the likelihood of developing issues such as anxiety, attention deficit hyperactivity disorder (ADHD), and poor behavioural habits (Sullivan et al., 2012; Vederhus et al., 2012).

Long-term alterations in cognitive function and neurological development have been linked to early life pain experiences (Victoria & Murphy, 2016). Increasing research (Ranger et al., 2015; Ranger & Grunau, 2015) suggests that cognitive abilities are diminished and the frontal and parietal cortical areas are thinned, resulting in impaired visual-motor skills and reduced cognitive function. In addition, early-life pain is associated with increased amygdala volume, which leads to the reprogramming of circuits underlying executive, cognitive, and emotional processing, resulting in far-reaching implications for behavioural and neuropsychiatric factors (Victoria & Murphy, 2016).

The endogenous pain modulation circuit, mediated by endogenous opioid peptides, is modified due to early-life pain (LaPrairie, 2009). Experiences of pain lead to increased levels of endogenous opioids and increased ß-endorphin and enkephalin, effects that continue into adulthood within the PAG (Victoria et al., 2013). This results in heightened excitability in the spinal cord's dorsal horn (Jankowski et al., 2014) and enhanced descending inhibition from the RVM (Zhang, Wang et al., 2010), leading to alterations in the opioidergic pathways that typically regulate pain management. As a result, adults exhibit diminished sensitivity to pain and decreased hypoalgesia (LaPrairie, 2009). The diminished sensitivity is further attributed to changes in neuronal excitability and potentiation of glutamatergic signalling in the dorsal horn, which leads to an enhanced excitatory response to pain, resulting in increased activity in pathways that favour nociceptive activity (Walker et al., 2015). Overall, experiences of pain in early life play a critical role in shaping neurodevelopment, with long-term implications for cognitive, emotional, and physiological functioning.

1.12 Clinical Implications and The Need for Early Intervention

Recognising and addressing pain early in life is critical, as timely intervention may prevent long-term consequences and improve outcomes across childhood. Past beliefs about infants' capacity to experience pain have had lasting effects on clinical care, and examining these misconceptions helps to explain current gaps in pain management. Historical misconceptions about infants' pain perception have contributed to delays in recognising and treating pain, resulting in inadequate pain management strategies in paediatric populations. Outdated beliefs, such as the notion that infants do not

experience pain similarly to older children or adults, or that pain is merely a reflexive response without emotional or cognitive involvement, have persisted in clinical settings. As noted by Bellieni (2012), these views have caused significant inconsistencies in pain management for this vulnerable population. Such misconceptions not only impede effective pain control but also limit research into the complexities of infant pain, leaving young patients without sufficient relief during procedures or hospitalisations.

Failure to adequately address infant pain may have lasting consequences that extend well beyond the immediate clinical setting. Insufficient analgesia during early-life procedures not only limits the effectiveness of pain management in subsequent medical interventions but may also predispose individuals to heightened pain sensitivity later in life (Weisman et al., 1998). Moreover, repeated exposure to untreated pain can induce maladaptive neuroplastic changes in the developing nervous system, contributing to chronic pain conditions or altered pain perception in adulthood. Together, these outcomes highlight a critical gap in both clinical practice and research, emphasising the importance of understanding how early-life pain experiences shape long-term pain processing and management.

There is a pressing need for research aimed at developing early intervention strategies to mitigate neuroplastic changes associated with early-life stress and pain (Jaffal, 2025). Prioritising the creation of prevention and management techniques tailored explicitly for infant pain (Harrison et al., 2010) can reduce the occurrence of untreated pain in this population. Additionally, implementing pain management protocols that account for infants' developmental stage and unique needs is essential to ensure appropriate analgesia (Atefeh, 2025). Addressing these challenges will not only improve the immediate quality of care but also help prevent long-term consequences, ultimately enhancing lifelong pain outcomes for paediatric patients (Eckstein Grunau, 2013).

1.13 Mechanisms of Cancer-Related Pain in the Paediatric Population

Understanding the mechanisms of cancer-related pain in children is essential, as it is a universal experience spanning the entire disease continuum from diagnosis through treatment and often extending into adulthood and survivorship (Lu et al., 2011). Cancer-related pain arises from various mechanisms, including the disease itself, due to tumour infiltration and subsequent inflammation (Laird et al., 2011), as well as medical treatments and procedures such as chemotherapy and radiation (Seretny et al., 2014). Early pain exposure is a significant lifetime event that has the potential to alter both structural and functional aspects of the nervous system (Title et al., 2024).

Cancer-related pain is a multifactorial sensory experience comprising both nociceptive and neuropathic components, affecting up to 75% of patients with advanced cancer (NT Contributor, 2019; Yoon & Oh, 2018). The tumour can induce pain through inflammatory infiltration and the subsequent release of pro-inflammatory mediators by cancer cells, leading to damage to sensory nerves (Mantyh et al., 2010). A central pro-inflammatory molecule involved in cancer pain is Tumour Necrosis Factor-α (TNF-α), a

pro-inflammatory mediator released by both cancer cells and immune cells, with studies demonstrating the efficacy of TNF-α antagonists in mitigating cancer pain (García-Domínguez, 2025; Sommer et al.,2001). TNF-α is consistently present in the tumour microenvironment, causing a downstream cascade of more inflammatory cytokines and the recruitment of leukocytes with pro-metastatic properties (Macciò & Madeddu, 2012; Powell et al., 2021). This mediator's pro-tumour role is further supported by its ability to lead to immunosuppression, with a vast number of studies indicating that it acts directly on tumours to boost pro-metastatic characteristics, including cancer stem cell generation, invasion, and resistance to therapy (Baram et al., 2021; Ben-Baruch, 2019; Martínez-Reza et al., 2017; Weitzenfeld et al., 2016). Therefore, as TNF-α is a key mediator of tumour progression and the microenvironment, it should be more heavily implicated in treatment plans for an increasingly targeted therapeutic approach (Maguire et al., 2021).

Furthermore, nerve growth factor (NGF) is a pro-inflammatory molecule secreted by cancer cells that promotes cancer-mediated nociceptive behaviour and axonal sprouting within the tumour microenvironment (Jimenez-Andrade et al., 2011). Existing research recognises the critical role of NGF in tumour pain, and it is well established that NGF antagonists can attenuate nociceptive behaviours by reducing peripheral and central sensitisation markers and simultaneously inhibiting further neuronal sprouting (Jimenez-Andrade et al., 2011).

Taken together, this research highlights the complex interactions between tumour-secreted factors and the progression of disease, as well as the persistence of nociceptive behaviours in paediatric cancer patients. Neuroimmune interactions can drive structural and functional changes that prime the developing brain for heightened pain sensitivity later in life, with long-term implications for emotional and developmental outcomes (Karshikoff et al., 2019; Nusslock et al., 2024). Given these profound effects, accurately assessing and managing pain in children with cancer is critical to minimise long-term impacts and support overall well-being (Hirata et al., 2025).

1.14 Clinical Assessment of Paediatric Cancer Pain: The Challenges and Emerging Approaches

Much uncertainty still exists regarding the clinical assessment of pain in paediatric cancer patients, making it a significant challenge in clinical practice. Evaluating pain in children, especially those with cancer, involves clinicians considering a broad range of factors, including the child’s developmental stage, age, cognitive understanding, and communication abilities. Many children, particularly those in younger age groups, cannot often articulate their pain effectively, making accurate pain assessment even more difficult (Duffy et al., 2019; Mathews, 2011). This communication gap necessitates that healthcare providers rely on indirect measures and observational methods, which can be subjective and may not fully capture the underlying physiological and neurological processes contributing to pain.

Despite recent advancements in technology, childhood pain, especially cancer-related

pain, has historically received relatively little attention in clinical practice, contributing to the underutilisation of emerging tools such as EEG for pain assessment. Although there has been some progress in this area, the scientific understanding of how pain is presented, processed, and treated in children remains insufficient, especially when compared to our understanding of pain in adults (Mathews, 2011). This knowledge gap highlights a critical need for further research to understand the nuances of paediatric pain better and develop effective strategies to address it (Slater et al., 2025).

However, the limited research on paediatric pain is often mirrored in the limitations of current animal models, which do not adequately replicate the developmental complexities that are crucial to understanding how early-life pain influences the nervous system. As Taylor and Ferrari (2023) pointed out, animal models often overlook the developmental context of paediatric pain, which is vital for understanding its long-term neurodevelopmental impact.

1.15 Limitations of Preclinical Models in Paediatric Cancer Pain Research

Despite a growing recognition of the long-term consequences of early life pain, there remains a significant gap in the development of appropriate preclinical models that address paediatric cancer pain. Many models primarily focus on adults and fail to replicate the unique neurodevelopmental context in paediatric cases (Runco et al., 2022). Investigations of CIPN in cancer and cancer survivorship focus on the use of treatments, such as surgery and chemotherapy and their impact on the maturing nervous system. However, this fails to account for the fact that patients who undertake these treatments also have tumours, and the interaction of tumours with chemotherapy, especially, is not understood. The principal challenge of the study of cancer survivorship in animal models is associated with technical challenges around implantation of cancers into rodent model systems and having to “cure” these animals of cancer so that survivorship and the impact of treatment can be assessed. The ethical use of animals in research, especially in the UK, means that unwarranted suffering is not tolerated. Therefore, tools need to be developed that permit the study of cancer's impact on the maturing CNS while also minimising distress in animals and ensuring the reproducibility of experimental measurements.

The principal challenge of studying cancer survivorship in animal models lies in the technical difficulties of implanting tumours into rodent models. Moreover, the need to “cure” these animals of cancer complicates the process of studying the long-term effects of cancer treatment on the nervous system, particularly when assessing the impact on the maturing CNS. Furthermore, the ethical considerations surrounding animal research in the UK and other jurisdictions also pose challenges. Ethical guidelines aim to minimise suffering, which necessitates the development of new experimental tools that can study the impact of cancer on the developing nervous system while ensuring that animals experience minimal distress. These advancements are crucial for reproducing experimental measurements and providing reliable data on how cancer and its treatments affect the developing CNS in paediatric patients (Cook et al., 2023).

1.16 Targeting Cancer with Diphtheria Toxin: A Promising Approach for Medulloblastoma Therapy

Diphtheria toxin (DT) has become notable as a novel anti-cancer agent due to its delivery of cytotoxic therapy. DT inhibits protein synthesis in host cells by inactivating elongation factor 2 (EF-2), ultimately causing cell death (Chaudhary & Pandey, 2020). DT consists of two primary domains: the A domain, which acts as the site of ADP-ribosyltransferase activity, and the B domain, which mediates receptor binding and translocation (Choe et al., 1992). Upon binding of the B domain to the DTR, also known as the heparin-binding epidermal growth factor-like growth factor receptor (HB-EGF receptor), the toxin-receptor complex is internalised, thereby facilitating the irreversible arrest of elongation factor 2, an essential component for protein synthesis. This process results in the inhibition of protein synthesis and ultimately leads to cell death (Murphy, 2011).

By employing this toxin's mechanism, whereby populations are genetically engineered to express the human DTR, thus facilitating an approach that enables the selective eradication of cancer cells (Yalcinkaya & Carhan, 2025). This presents a promising novel therapeutic strategy that spares normal tissues. A critical feature of this model is that mice do not naturally express the DTR (Saito et al., 2001), rendering them inherently resistant to the cytotoxic effects of DT unless genetically modified to express the human receptor. Therefore, by leveraging this species-specific restriction, the development of a highly selective model is enabled, permitting targeted ablation of specific cell populations, such as tumour cells, at controlled stages of the disease. This cell-specific approach provides precise control over cell loss, enabling the study of long-term neurological sequelae following ablation.

The clinical appeal of the DT/DTR system in model development, when compared to other experimental tools, lies in its high specificity, potency and modular design, making it adaptable to a range of cancer subtypes (Alouf, Ladant, & Popoff, 2015; The Native Antigen Company, 2019). Alternative approaches include Cre-dependent apoptotic genes, optogenetic and irradiation-based tumour methods, which, unlike the DTR/DT model, may cause a variety of off-target effects (Malogolovkin et al., 2022; Moll et al., 2022; Pouget et al., 2018), lack precision or do not achieve cell-autonomous specificity. Given the irreversible and exceptionally specific nature of the DTR/DT system, it is particularly valuable for investigating the long-term effects of tumour ablation, allowing for the study of long-term outcomes, including cognitive, behavioural, and psychological effects following cell ablation. However, unlike chemotherapeutic or radiotherapeutic interventions, the DTR/DT model does not induce cumulative DNA damage or modify the tumour microenvironment, as observed in other cancer treatment modalities (Bai et al., 2024;Li et al., 2024). Therefore, this model provides only a specific aspect of tumour cell death rather than capturing the full complexity of therapeutic responses.

Overall, the precision and high specificity of the DTR/DT system render it a superior tool for the development of a medulloblastoma model that accurately mirrors survivorship scenarios, thereby facilitating the investigation of long-term treatment-related effects.

It is essential to focus on developing DT-based immunotoxins to create medulloblastoma models for studying the long-term effects of chemotherapeutic interventions. Improving this system will enable researchers to monitor changes over extended periods, assessing delayed or progressive effects (Mafalda et al., 2017), such as cognitive decline, alterations in neural plasticity (Das et al., 2020), and shifts in pain sensitivity (Was et al., 2022).

Furthermore, direct ablation of tumour cells at particular developmental or disease stages will facilitate the comparison between early and late therapeutic interventions, evaluating their impacts on behavioural and neurocognitive outcomes. In this way, by engineering murine cells to express DTR, this model acts as a powerful controllable research tool for studying the long-term effects of medulloblastomas and therapeutic interventions.

1.17 Research Aims

This project aims to express diphtheria toxin receptors in medulloblastoma cell lines as a tool for future in vivo research exploring the late effects of cancer in survivors. The primary objective is to develop a novel, growth-manageable form of medulloblastoma by stably transfecting the diphtheria toxin receptor into medulloblastoma cells. By utilising representative and well-characterised human medulloblastoma lines typically used in preclinical studies, DAOY and ONS-76 (Castellino, De Bortoli, et al., 2007), the study will evaluate their response to diphtheria toxin in 2D and 3D culture systems. Establishing this tool will enable future in vivo research, allowing for the complete ablation of implanted cancer cells and the systematic study of long-term neurological sequelae and consequences.

1.18 Research Hypothesis

Engineering medulloblastoma cell lines ONS-76 and DAOY to express the DTR will enable specific, tuneable tumour cell ablation with minimal off-target effects, thereby allowing for the development of more precise experimental models that replicate survivorship scenarios, supporting the investigation of long-term treatment-related outcomes, including cognitive deficits, behavioural alterations, neurodevelopmental disruption and persistent pain.

2.0 Methods

All procedures were carried out in a Class II biosafety cabinet to prevent contamination. All surfaces were disinfected with Chemgene prior to use, and all used consumables were disposed of in accordance with biosafety guidelines. Personal protective equipment, including lab coats, gloves, and eye protection, was worn at all times.

2.1 Cell culture maintenance for ONS-76 and DAOY Cells

All cells were cultured and maintained under standard mammalian cell culture conditions, in a humified incubator at 37 °C with 5% CO_2 to support optimal growth and viability. ONS-76 were maintained and cultured in RPMI 1640 medium (Sigma-Aldrich, R8758) with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, F7524). Cells were passaged twice weekly at a 1:10 ratio for several weeks to support growth and viability.

Before passage, microscopes were used to assess the cells' confluency and overall health. Once they reached 70% confluency, they were passaged. For passaging, culture medium was aspirated from the T-75 tissue culture-treated flasks (Corning, 430641U), and the cells were washed in 5 mL of Hanks Balanced Salt Solution (HBSS, Gibco,14170) to remove any cells in suspension. Following HBSS aspiration, 2 mL of trypsin-EDTA (Sigma, LH-SIG2044) was added to cover the flask's surface, ensuring all adhered cells were covered. The flask was incubated at 37 °C with 5% CO_2 for 5 minutes to encourage cell detachment. Following gentle tapping of the flask to encourage cell detachment, the detachment was confirmed by viewing it under the microscope.

Following cell detachment, the trypsin medium was aspirated from the cells into a 15 mL conical tube. RPMI + 10% FBS medium for ONS and DMEM (Sigma-Aldrich, D6429) + 10% FBS for DAOY were used to wash the flask, and the solution was aspirated into the conical tube containing the trypsin medium. The tube was centrifuged at 100 x g for 5 minutes at room temperature to pellet the cells. Following centrifugation, the medium was removed without disturbing the pellet, and the cells were resuspended in the appropriate medium. Cells were suspended in the appropriate medium volume, with 1 mL of this being aspirated into a new T-75 flask with the correct volume of fresh RPMI medium to ensure a 1:10 ratio of cells. The flasks were gently swirled to ensure even distribution and incubated at 37 °C with 5% CO_2.

Cell growth was monitored regularly, and each passage was documented and labelled with the passage number and date.

2.2 Cell counting

Cells were counted to calculate the number of cells/mL. Following pelleting of the cells, as previously described in cell maintenance steps, a 1:1 Dilution of 10 µL cells to 10µL of Trypan Blue (Gibco, 15250061) was generated in a 1.5mL tube. Following gentle pipetting to mix, 10µL of the solution was added to a haemocytometer chamber and

placed under the microscope. Cells were counted in the four outer quadrants of the grid, with cell concentration counted using the following formula, as previously described by Roper and Coyle (2022).

$$Total\,cells/ml = \frac{Total\,cells\,counted}{Number\,of\,squares\,counted} \times 2\,(trypan\,blue\,dilution\,factor) \times 10{,}000$$

Equation: This equation was used to calculate cell concentration after counting using a haemocytometer.

2.3 Prestoblue assay on ONS-76 and DAOY Cell line

The Prestoblue assay (ThermoFisher Scientific) was used to assess changes in cell viability and proliferation. The PrestoBlue® Cell Viability Reagent quantifies cell proliferation by utilising a resazurin-based solution, which, upon entering a living cell, is reduced to resorufin, a highly fluorescent compound. Therefore, cellular viability can be measured by the fluorescence signal emitted.

2.3.1 Cell Seeding and Incubation

On Day 0, ONS-76 and DAOY cell lines were seeded at a density of 1.5 x 105 cells/mL in triplicate within a 96-well black plate. Cells were cultured in 100 µL of RPMI + 10% FBS for ONS and DMEM + 10% FBS for DAOY. The plate was incubated for 6 hours at 37°C with 5% CO_2 to allow cell attachment and initial growth.

2.3.2 Prestoblue Treatment and Fluorescent Measurements

Subsequently, the plate was analysed using a BMG FLUOstar OMEGA microplate reader (BMG Labtech) to assess metabolic activity. Each day, three wells containing cells were tested alongside two controls comprising Prestoblue® with culture medium to account for background fluorescence.

On Day 0, ONS-76 and DAOY cells were seeded in triplicate in 100 µL of RPMI + 10% FBS for ONS and DMEM + 10% FBS for DAOY. The plate was incubated for 6 hours at 37 °C with 5% CO_2 to allow cell attachment and initial growth. From Days 1 to 6, 100 µL of media was removed from each well with cells. Each day, a solution of 50 µL of PrestoBlue+450 µL RPMI and 50 µL + 450 µL DMEM was prepared, with 100 µL distributed to each well alongside two controls comprising Prestoblue® with culture medium to account for background fluorescence. The plate was then incubated at 37 °C with 5% CO_2 for 45 minutes to allow the reagent to interact with metabolically active cells within the well.

Subsequently, fluorescence was measured using a BMG FLUOstar OMEGA microplate reader at excitation and emission wavelengths of 560 and 590 nm, respectively. Fluorescence readings from each well were recorded. Data collected from daily

fluorescence measurements were used to calculate cell viability and to generate growth curves for ONS-76 and DAOY cell lines. The control value was subtracted from the cell fluorescence values to correct for background fluorescence. All cell fluorescence values and controls were performed in triplicate to ensure reproducibility and validity.

2.4 Preparing DAOY and ONS-76 Cell Line Stocks

Cells were frozen as stocks to preserve our valuable cell lines for increased availability for further experimental procedures (Miyamoto et al., 2018). Prior to freezing, cells were maintained under previously listed culture conditions and grown to at least 70-80% confluency to optimise cell health and recovery upon thawing.

2.4.1 Thawing Freezing Medium

CellBanker2 (Ambsio,11914) was thawed in a 37°C water bath while the cells were being prepared. Once thawed, the medium was gently mixed to ensure uniform distribution and kept at room temperature. The careful thawing of CellBanker2, ensuring it did not overheat, and maintaining close monitoring of the water bath temperature were crucial for maintaining consistency in cryopreservation, which supported the development of a reliable stock of ONS-76 and DAOY cell lines.

2.4.2 Harvesting Cells

The medium from the T-75 flask containing cultured cells was aspirated and disposed of. Five millilitres of HBSS was added to wash the cells, which were then aspirated into a 15 mL conical tube to minimise cell loss during the washing stages. To detach the cells from the flask surface, 2 mL of trypsin was added, swirled to cover the base of the flask, and incubated at 37°C for five minutes.

Following incubation, gentle tapping of the flask was employed to encourage cell detachment, which was confirmed using a microscope. 2 mL of complete medium were added to the flask to neutralise the trypsin, and this mixture was transferred to a 15 mL Falcon tube containing the first wash. The T-75 flask was rewashed with 2 mL of HBSS, and the wash was discarded into the Falcon tube. After centrifugation at 800 rpm for 5 minutes, the supernatant was discarded, while the cell pellet was retained.

2.4.3 Counting Cells

Cell pellets were resuspended in 1 mL of complete medium and counted using the previously described protocol. Following a further five-minute centrifugation, the pellet was retained.

2.4.4 Freezing and Storage

The pellet was resuspended in CellBanker2 at a 1 × 10^6 cells/mL density. 500 µL of cell suspension was aliquoted into Cryotubes and stored at -80°C for short-term storage

until required for future experimental procedures.

2.5 Determination of Optimal Puromycin Concentration and Kill Curve Analysis for DAOY, and ONS-76 Cell Lines

A dose-response kill-curve assay was conducted to determine the optimal puromycin concentration for effective selection of cells which stably express the puromycin resistance gene, ensuring only transfected cells are utilised for downstream experiments. Initially, a concentration range of 0-10 µg/mL was established. Cells were seeded in a 96-well plate at a seeding density of 1 × 105 cells/mL and incubated for 24 hours before the culture medium was replaced with puromycin-containing medium at increasing concentrations.

Over the course of five days, cells were monitored for morphological changes and confluency using a microscope. Initial observations indicated that concentrations above 20 µg/mL caused complete and rapid cell death, suggesting a narrower range was needed to determine a more precise initial toxicity threshold.

Following this, a refined concentration was tested between 0 and 20 µg/mL, using doses of 0, 2, 5, 10, 15, and 20 µg/mL. Cells were monitored again for changes and growth. At ≥2 µg/mL, complete cell death was observed across DAOY and ONS-76 cell lines, indicating that a lower concentration was required.

To refine the selection process further, a lower concentration was tested with puromycin concentrations between 0 and 2 µg/mL (0, 0.25, 0.5, 1, 1.5, and 2 µg/mL). Cells were observed morphologically, assessed visually for viability under a microscope, and further confirmed quantitatively with PrestoBlue cell viability assays performed on Days 3 and 5. The PrestoBlue assay quantitatively measures metabolic activity in surviving cells, enabling an objective assessment of cell viability across various concentrations.

Using viability readings from the Prestoblue Assay, we generated a puromycin kill curve for each cell line. This curve illustrates the dose-dependent effects of puromycin, enabling us to identify the minimum effective concentration needed to eliminate non-resistant cells.

2.6 Transfection of the diphtheria toxin receptor using the Lentivirus vector

ONS-76 and DAOY cells were cultured in RPMI+10% FBS and DMEM with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin, respectively, under standard conditions (37°C, 5% CO_2).

A commercially prepared lentiviral vector (Figure 2), encoding the Diphtheria toxin

receptor along with a Green Fluorescent Protein (GFP) reporter gene, was provided in a ready-to-use format. The viral stock was thawed on ice and subsequently diluted in culture medium to attain a multiplicity of infection (MOI) of 1 prior to the transduction of the ONS-76 and DAOY cell lines.

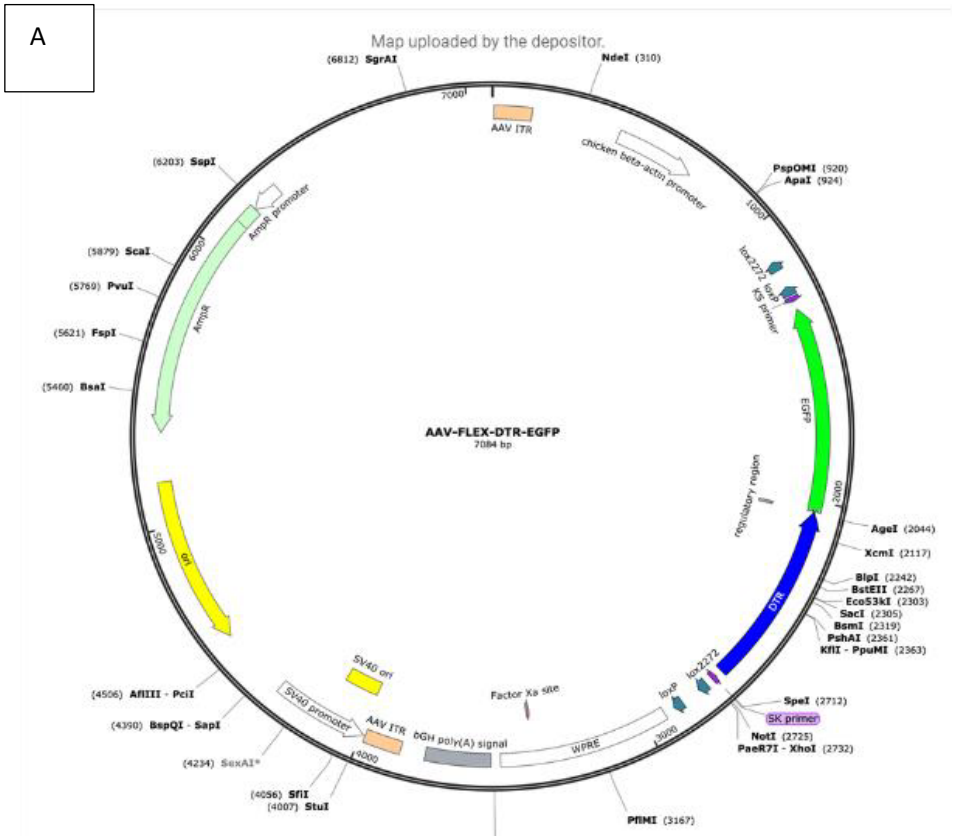


Figure 2A, Schematic representation of the lentiviral construct for DTR and GFP reporting, used to transduce ONS-76 and DAOY cells. The plasmid contains the diphtheria toxin receptor under the control of a constitutive promoter, along with green fluorescent protein, which has been reported to show transduction efficiency.

B

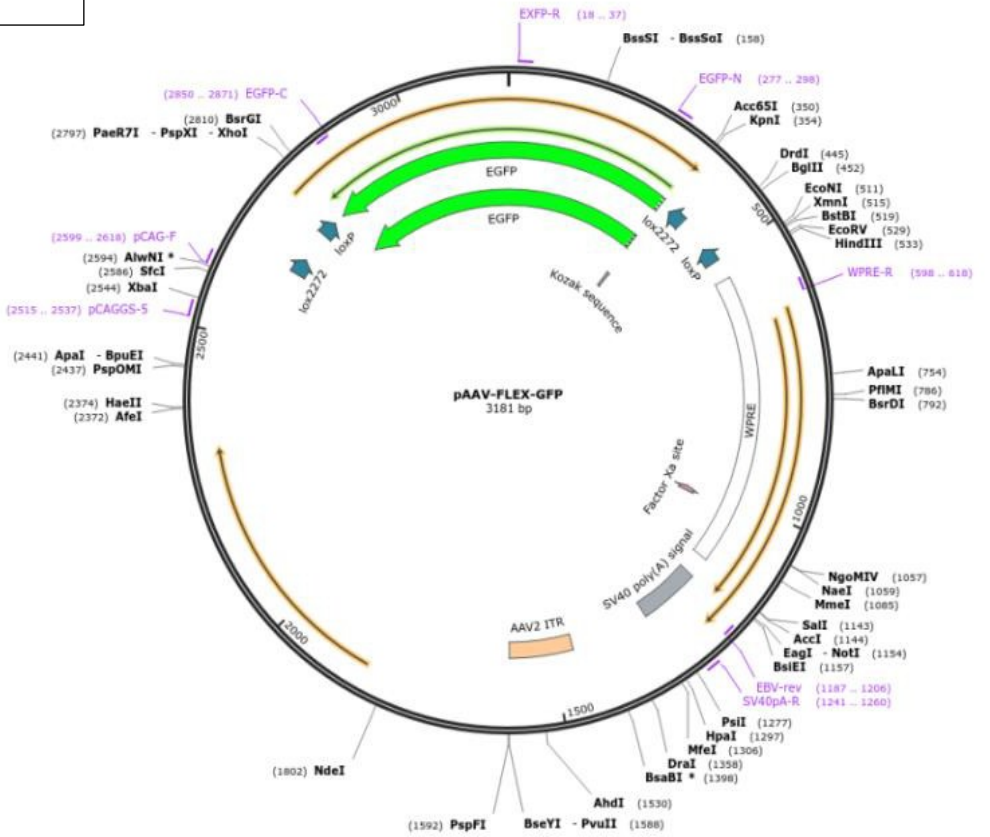


Figure 2B Schematic representation of the GFP-only control plasmid, used to assess baseline transduction efficiency.

ONS-76 and DAOY cells were used as a baseline condition to determine optimal conditions for lentiviral transduction. The following transduction conditions were trialled.

Condition	**Experimental Changes**
Condition 1	MOI1+Polybrene+Lentivirus+45 Minute Spin Inoculation
Condition 2	MOI2+Polybrene+Lentivirus+45 Minute Spin Inoculation
Condition 3	MOI1+Polybrene+Lentivirus
Condition 4	MOI2+Polybrene+Lentivirus
Condition 5	MOI1+Lentivirus
Condition 6	MOI2+Lentivirus

Table 1 displays the experimental conditions tested to optimise stable expression parameters of DTR and GFP for the ONS-76 and DAOY cell lines. Cells were transduced under six different conditions, varying MOI, use of polybrene, and spin inoculations. Conditions 1-4 included polybrene, which facilitated lentiviral entry into the cells; conditions 1 and 2 employed spin. Inoculation was used to promote entry further and increase transduction efficiency. MOI 1 and 2 were tested across all conditions to evaluate the effects of viral load on gene delivery.

After removing the culture medium from ONS-76 and DAOY cells, 2 mL of transduction medium was used per well to ensure uniform coverage. Cells were incubated overnight at 37°C in a humidified CO_2 incubator. Following incubation, the transduction medium was aspirated and replaced with a complete culture medium in each well, and the wells were then incubated overnight.

On Day 2, expression was assessed using GFP fluorescence under a fluorescence microscope. Cells were examined under excitation and emission wavelengths specific to GFP (488nm and 510nm, respectively). Successful transduction was confirmed by the presence of green fluorescence within the cytoplasm of ONS-76 and DAOY cells. The percentage of GFP-positive cells' coverage was used to indicate transduction efficiency in measuring DTR expression.

Antibiotic selection with puromycin created a stable population of DTR-expressing ONS-76 and DAOY cells. Before exposing cells to puromycin, the optimal concentration was determined. This was carried out by treating non-transduced cells with varying concentrations of puromycin and assessing cell viability daily using PrestoBlue assays at 24-hour intervals. The lowest concentration that allowed for complete cell death was selected. This predetermined optimal concentration of puromycin replaced the DMEM medium for the cells and was reapplied every 48 hours to maintain concentrations. Following successful selection, the stable population was transferred to a T-25 flask for continued growth and observed daily to assess cell viability and morphological changes.

After determining that the optimal transduction condition was MOI 1 + Polybrene + 45-minute spin inoculation, the same optimised protocol for transduction was applied to all downstream experiments to generate transfected cells both in culture and in stock. The same conditions and method of transduction and assessment were used, and successful transduction was confirmed by GFP fluorescence. Following successful selection, the stable population was transferred to a T-25 growth for continued growth and observed daily to assess cell viability and morphological changes.

2.7 Fluorescence-Activated Cell Sorting of DTR-Expressing Clones

Fluorescence-activated cell sorting (FACS) was employed to isolate the top 20% of DTR-expressing ONS and DAOY cells, characterised by their GFP fluorescence levels. Cells were initially prepared as a single-cell suspension in HBSS (Ca^{2+}/Mg^{2+} free) with 1% BSA (Bradford, 1976) and 1 mM EDTA to prevent aggregation. Utilising a flow cytometer equipped with an excitation laser for GFP at 488nm, the cells were analysed for GFP

expression. The top 20% of cells exhibiting the highest GFP fluorescence were gated and sorted to enrich for cells with the highest DTR expression. The sorted cells were collected under sterile conditions for downstream experiments, ensuring a highly purified subpopulation based on GFP fluorescence.

2.8 Spheroid Generation of DAOY and ONS-76 Cell Lines

Neurosphere media were prepared using DMEM/F12 as a growth medium for both cell lines. The composition for 200 µl per well used in Ultra Low attachment 96 well plates (Corning, 7007) is as follows:

Table 2 Preparation of the complete culture medium for 3D spheroid generation, showing the constituent components, stock concentrations, final working concentrations, and corresponding volumes.

Component	Stock Concentration	Final Concentration	Volume (for 7mL)
DMEM/F12 (Gibco, 11320)	1X	1X	7,000 µL
B-27 (Gibco, 17504044)	50X	1X	140 µL
N-2 (Gibco, 17502048)	100X	1X	70 µL
Heparin (Stem Cell Technologies, 07980)	2 mg/Ml	2 µg/mL	18 µL
EGF (EGF; Gibco, PHG0315)	0.1 mg/mL	20 µg/mL	1.5 µL
FGF (bFGF; Gibco, PHG0266)	0.1 mg/mL	10 µg/mL	1 µL

Once at 80% confluency, DAOY and ONS-76 cells were detached using trypsin and pelleted at 123 × g for 5 minutes. The cell pellet was then washed with 10 mL HBSS and centrifuged again under the same conditions twice to remove residual FBS serum. The final pellet was then resuspended in 3 mL of neurosphere medium.

Cells were counted using Trypan Blue staining and a haemocytometer, and the pellet was diluted in the correct medium to give a seeding density of 2500 cells/mL for DAOY and 1250 cells/mL for ONS-76 cells.

200 µL of cell suspension was seeded into each of the 32 inner wells, and 200 µL of HBSS was added to the outer wells to prevent evaporation, using a multichannel pipette. Confirmation of successful seeding was achieved by visual inspection under a light microscope. Plates were then incubated at 37°C in a humidified atmosphere with 5% CO_2 for 4 days. Spheroid formation was visually confirmed on Day 4, and images were taken.

2.9 Preparation of Diphtheria Toxin Dilutions

Diphtheria Toxin (DT, The Native Antigen Company, DIP-TNL-100) was received as a lyophilised powder and stored at -20 °C. For experimental use, serial dilutions were prepared, allowing for DT to be thawed on ice upon use. For the initial dilution, DT was reconstituted in phosphate-buffered saline (PBS) to generate a 1mg/mL stock dilution. Serial dilutions were prepared to achieve final working concentrations specific to each experimental condition using RPMI+FBS+PenStrep for ONS-76 Cells and DMEM+FBS+PenStrep.

For 2D culture assays conducted in a 96-well plate, the following cells were Initially treated with DT at final concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 ng/mL, as seen in Table 2, to create a baseline guide for concentrations specific to each cell line.

Table 3 Details the calculated volumes of intermediate DT stock and complete cell culture medium needed for each dilution. These dilutions were used to determine dose-response curves in both ONS-76 and DAOY cells.

Final DT Concentration (ng/mL)	Volume of 1 µg/mL Intermediate (µL)	Volume of Medium (µL)
0.0	0.00	1000.00
0.2	0.20	999.80
0.4	0.40	999.60
0.6	0.60	999.40
0.8	0.80	999.20
1.0	1.00	999.00

Following the analysis of kill-curves generated from the 2D plate data, which indicated that DAOY cells exhibited relative resistance to DT at a concentration below 1.0 ng/mL, a refined concentration series of 0, 0.4, 0.8, 1.2, 1.6, and 2.0 ng/mL was employed for DAOY cells, as seen in Table 3. Conversely, ONS-76 cells remained at the initial concentration.

Table 4: To expand the dose-response analysis beyond 1.0ngmL, additional DT concentrations were explicitly prepared for DAOY.

Final DT Concentration (ng/mL)	Volume of 1 µg/mL Intermediate (µL)	Volume of Medium (µL)
1.2	1.20	998.80
1.6	1.60	998.40
2.0	2.00	998.00

2.10 Cell Seeding and DT Treatment in 2D Culture

For this section, the term “all cells” refers to the following:

- ONS-76
- ONS-76 DTR Expressing
- ONS-76 GFP Expressing
- DAOY
- DAOY DTR Expressing
- DAOY GFP Expressing

All cells were seeded as previously described into separate flat-bottom 96-well tissue culture plates at a density of 3 × 105 cells per well. Following overnight incubation to allow for cell adherence to the plate, the culture medium was aspirated and replaced with DT-containing medium at the given concentrations. Cells were then incubated for 48 hours.

2.11 Assessment of Percentage Cell Viability in 2D Plate

Following 48 hours of DT treatment, cells were assessed for proliferation and viability using PrestoBlue as previously described using a BMG FLUOstar OMEGA microplate reader at excitation and emission wavelengths of 560 and 590 nm, respectively. Results were normalised using a blank reading of solely PrestoBlue+Media and recorded as percentage viability using the given equation.

Cell viability (%) = (X treatment − X blank)/(X control − X blank) × 100% (where, X = fluorescence units)

2.12 Cell Seeding and DT Treatment in 3D Spheroid Cultures

All cells were seeded into separate 96-well ultra-low attachment spheroid plates, using 32 central wells for experimental conditions. All ONS-76 cell conditions and DAOY cell conditions were plated at seeding densities of 250 cells/mL and 500 cells/mL, respectively, in neurosphere media.

On Day 4, to facilitate spheroid formation, half of the culture medium was aspirated and replaced with DT-containing medium at the refined concentration ranges shown in Table 2. The cells were then incubated for an additional 48 hours.

2.13 Assessment of Percentage Cell Viability in 3D Plate

Following 48 hours of incubation with DT-containing medium, viability was assessed using the CellTiter-Glo® 3D Cell Viability Assay (Promega, G9683). Reagent was added

to the well with a 1:2 dilution in neurosphere media. The plates were shaken on an orbitaK incubator (Stuart, SI500) at 250 rpm for 5 minutes to induce cell lysis and then incubated at 37 °C with 5% CO_2 for 25 minutes. Following this, luminescence was measured using the BMG FLUOstar OMEGA microplate reader, and values were normalised using a blank of media + CellTiter-Glo to calculate percentage cell viability using the previously described equation.

2.14 Statistical Analyses

All data were analysed using GraphPad Prism 9.5.1 software. The data were tested for normality with the Shapiro-Wilk test and analysed using parametric or non-parametric tests as appropriate. Puromycin kill curves were generated using non-linear regression (inhibitor vs. response, variable slope), with IC50b values determined for each cell line. To test variations in basal growth comparisons between wild-type and DTR-expressing lines, an unpaired two-tailed t-test was utilised. Spheroid growth was analysed using a one-way ANOVA, with Tukey's post hoc test. To study the cytotoxic effects of DR, a one-way ANOVA with Tukey's or Šídák's post hoc test for normally distributed data was used, or Kruskal-Wallis when normality assumptions were not met. Furthermore, the correlation between puromycin concentration and cell viability was assessed using Pearson's correlation or Spearman's correlation, depending on whether the normality assumptions were met. Statistical significance was set at $p < 0.05$.

3.0 Results

The primary objective of this project was to develop a successful and reliable model for selective cell ablation in medulloblastoma models, specifically in SHH subtypes ONS-76 and DAOY, by stably expressing DTR receptors. To accomplish this, our initial phase involved generating puromycin kill curves for both cell lines to precisely identify suitable antibiotic concentrations for the selection of stable transduced populations. The subsequent phase focused on optimising the conditions for lentiviral transduction of the DTR plasmid, utilising polybrene, varying MOI, and spin inoculation to ensure consistent expression of DTR and GFP constructs in both cell lines. A robust system for culturing 3D spheroids was established using ultra-low attachment plates, enabling a more physiologically relevant model for assessing growth dynamics and the effects of transgene expression.

Once an appropriately optimised model was developed, the functional impact of diphtheria toxin (DT) treatment on transduced cells was evaluated in both 2D and 3D cultures to assess effects on basal growth and to determine the specificity of DT-induced cytotoxicity. Collectively, these experiments provide a framework for further research employing targeted cell populations within specific medulloblastoma subtypes using the DTR/DT system.

3.1 Puromycin Reduces Viability of Wild-Type ONS and DAOY in a Dose-Dependent Manner

To establish an effective method for antibiotic selection, puromycin was applied at various concentrations ranging from 0 to 2.0 µg/mL to generate a kill curve, which served as a prerequisite for generating stably transfected cell clones in this study. This step ensures that only cells successfully transfected with GFP are allowed to survive. The appropriate concentration of puromycin required to eliminate wild-type cells was first determined, a critical step for maintaining selective pressure in downstream experiments. Cell viability was quantified using PrestoBlue Cell Viability Reagent after 48 hours of exposure to puromycin. Data were fitted to a non-linear regression model in the form of Inhibitor vs. response, using a variable slope.

For ONS cells, the non-linear regression revealed a complete loss of viability at high concentrations of puromycin, with an R^2 value of 0.9193, indicating a robust, steep dose-dependent cytotoxic effect as shown in Figure 3A. Conversely, DAOY cells exhibited a different profile, demonstrating higher tolerance to puromycin and requiring higher doses for an equivalent cytotoxic response. Regression analysis of DAOY cells yielded an R^2 value of 0.8273, with a notably steeper slope in the dose-response curve (see Figure 3B). Therefore, the R^2 values obtained were 0.9193 for ONS-76 and 0.8273 for DAOY, reflecting a robust correlation between cell viability and puromycin.

These findings support and validate the use of puromycin to assess cytotoxic responses across cell lines. A direct comparison of IC_{50} values revealed that ONS cells are

approximately 12 times more sensitive to puromycin than DAOY cells (0.1087 µg/ml vs. 1.291 µg/ml). This highlights the significant differences in cell line responses and the need to determine individual kill curves rather than assuming uniform responses among similar cell types. Based on these analyses, working concentrations of 1.8 µg/ml for ONS cells and 3.4 µg/ml for DAOY cells were selected to maintain antibiotic pressure in subsequent experiments.

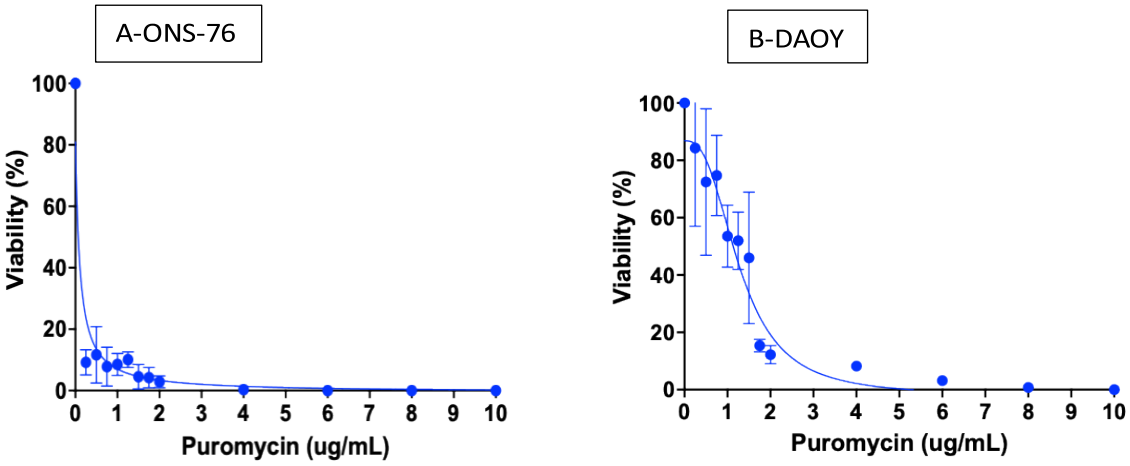


Figure 3 dose-response data were modelled using a four-parameter logistic curve (inhibitor concentration vs. response, variable slope) with ONS (Figure 3A) and DAOY (Figure 3B). The IC50 values obtained were 0.1087 µg/ml for ONS (R^2=0.9193, DF=29, n=4) and 1.291 µg/ml for DAOY cells (R^2=0.8273, DF=29, n=4). These kill curves form the statistical basis for determining the optimal puromycin concentration, which effectively eliminates un-transfected cells while preserving transfected populations. Data are presented as mean ± SD.

3.2 Optimisation for DTR/DT Transfection Conditions in ONS-76 and DAOY Cells

To establish an effective transduction method for DTR gene transfection, we carried out optimisation experiments directly on ONS-76 and DAOY medulloblastoma cell lines. To guide our transduction parameters, we utilised established conditions from previous studies involving neural and adherent cell types (Naldini et al., 1996).

Key variables included the Multiplicity of Infection (MOI), the use of polybrene, and spin inoculation. MOI denotes the ratio of infectious units to each available cell for transfection, thereby playing a crucial role in optimising viral infectivity (Zhang et al., 2004). Based on prior literature by Amado and Chen (1999), which demonstrates that excessively high MOI values are counterproductive and may reduce the number of transduced cells. The addition of polybrene was selected due to its capacity to enhance transfection efficiency by decreasing electrostatic repulsion between the virus and the cell surface, thereby facilitating interaction (Davis et al., 2002). Furthermore, spin inoculation can be employed to augment viral uptake (Guo et al., 2011).

To evaluate the effectiveness of various conditions, GFP fluorescence was utilised as a

marker as previously described, with GFP expression confirmed via fluorescence microscopy 72 hours post-transduction. Among the tested conditions, as outlined in Table 1, the combination of MOI 1, polybrene, and spin inoculation yielded a robust and consistent level of GFP expression in ONS-76 and DAOY cell lines. As depicted in Figure 4C, an increase in widespread and intense fluorescence indicates a high proportion of successfully transduced cells. Increased fluorescence is observed in Conditions 1-2, in both Figure 4A and 4B, with the optimal condition shown by the highest visual level of fluorescence in Figure 4A-1 and 4B-1, corresponding to Condition 1 as listed in Table 1. Based on these results, the condition using MOI 1, polybrene, and spin inoculation was identified as the optimal setup and was used for subsequent experiments with ONS-76 and DAOY cell lines. This optimisation step was crucial in ensuring a reliable and reproducible transfection model for the DTR system using lentiviral vectors.

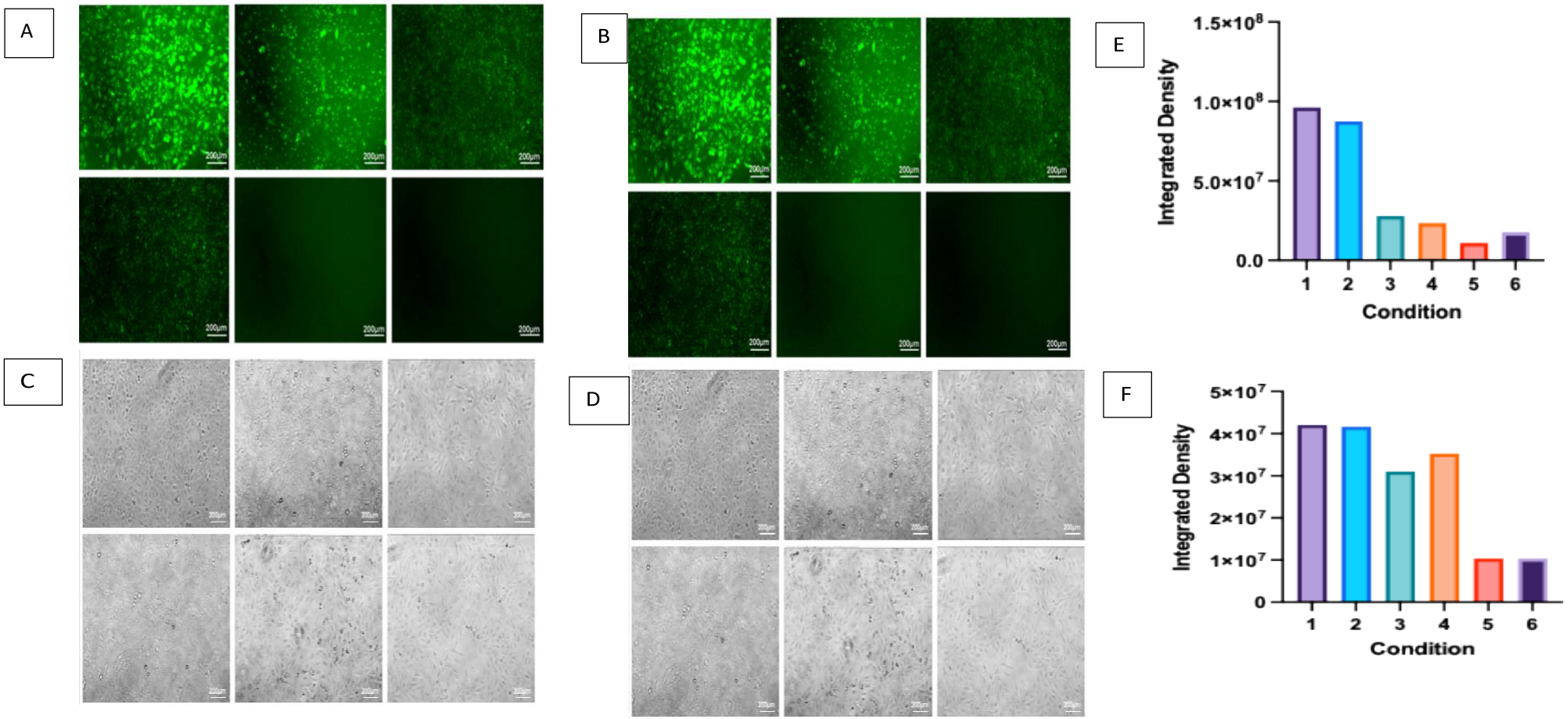


Figure 4A shows representative fluorescence microscopy images of ONS-76 cells transduced under six different conditions, taken 48 hours post-transduction, with the most notable fluorescence seen in Condition 1. Figure 4B displays corresponding images of DAOY cells under the same conditions, showing a similar pattern of GFP intensity. (C) and (D) represent the corresponding brightfield images for ONS-76 and DAOY cells, respectively. (E) and (F) show the corresponding graphical representation of integrated GFP fluorescence density for ONS-76 and DAOY, respectively, as quantified using ImageJ. Each bar represents the mean integrated density for each condition, with higher values indicating greater transfection efficiency. Condition 1, MOI1 with Polybrene and a Lentiviral 45 Minute Spin Inoculation, yielded the highest GFP expression in both cell lines and was therefore chosen for subsequent experiments

3.3 Enrichment of DTR-Expressing DAOY and ONS-76 Cells by FACS Sorting

Sorting of DTR-expressing DAOY and ONS-76 cells was performed using a BD FACSAria system, with GFP fluorescence used to identify DTR-expressing cells relative to non-transduced populations, allowing only highly enriched GFP clones to be utilised in downstream experiments. Gates were optimised with non-transduced DAOY and ONS-76 parental lines as negative controls. The gating strategy and representative sorting plots are shown in Figure 6. A pooled population of the top 20% GFP-positive fractions was isolated and collected in complete medium and immediately returned to culture. Following recovery, GFP-positive populations were expanded, counted, and preserved in CellBanker2 using the same procedure as described in Section 2.4.4.

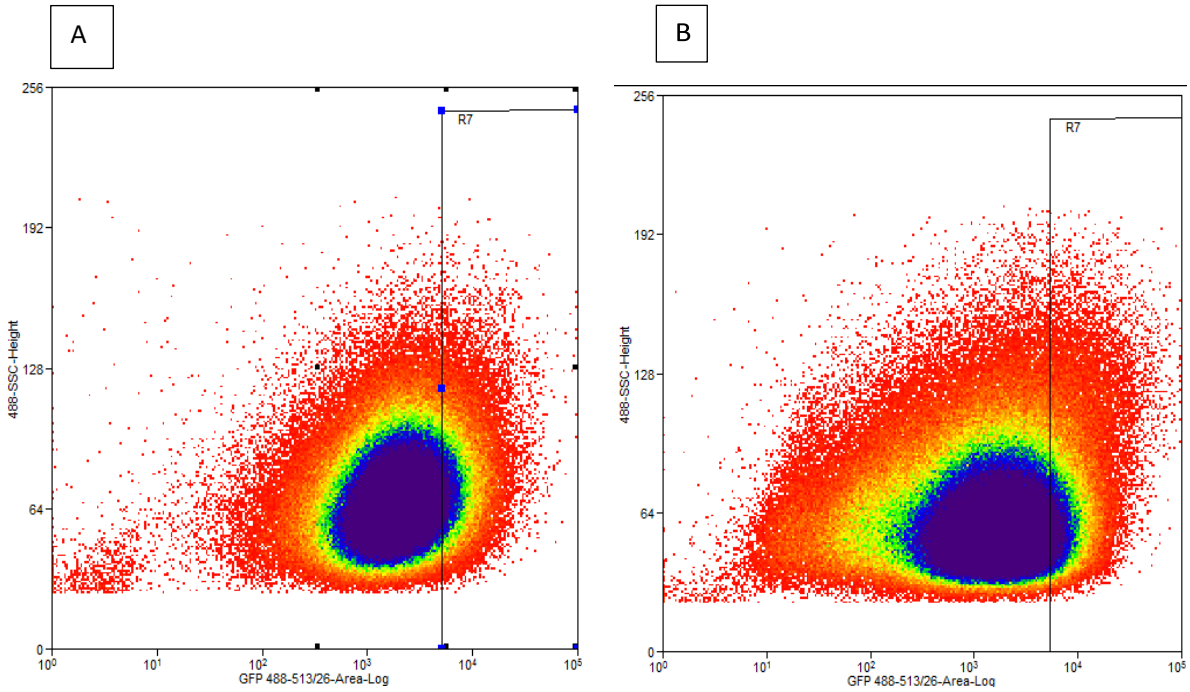


Figure 6 illustrates dot plots displaying the distribution of GFP fluorescence intensity in ONS-76(A) and DAOY (B) DTR-expressing cells in relation to side scatter. Gates were established to identify the top 20% of DTR populations (indicated by a boxed region) in comparison to negative controls. Areas with high cell density are represented by a colour gradient ranging from blue to red, with blue signifying the highest density of positive GFP-expressing cells.

3.4 Optimisation of a 3D spheroid model of ONS76 and DAOY medulloblastoma

The first step in developing a three-dimensional medulloblastoma model was to generate an adequate spheroid model that facilitates the creation of a more physiologically relevant environment (Yun et al., 2024), thereby enhancing the study's

validity and reliability. The use of spheroids better recapitulates the complex tumour microenvironment and cell-cell interactions when compared to standard 2D monolayer cultures (Mangani et al., 2025). As a result, this model will be invaluable for further applications that will enable us to use this model directly in 3D tumours, rather than 2D monolayer cultures. We chose to use a spontaneous formation method with non-adherent, ultra-low attachment (ULA) round 96-well plates. These plates prevent spheroids from adhering through a covalently bonded hydrogel layer, which provides a hydrophilic, negatively charged environment that allows cells to remain suspended and naturally aggregate, thereby mimicking in vivo conditions (Gómez-Mercader et al., 2024).

On day 0, cell lines were seeded into a 96-well ULA plate containing neurosphere media, typically forming aggregates by day 1. Once spheroids reached their optimal size with a diameter of 250–350 µm, as outlined in previous research by Roper and Coyle (2022), we considered these to be optimised for experimental work. Before transfection and viral work, we tested the established ONS-76 and DAOY medulloblastoma cell lines to ensure they could form sufficient cell aggregates, as described by Ivascu and Kubbies (2007), over a 7-day period.

Figure 5 illustrates the increased growth over this period; however, we observed unexpectedly high growth beyond day 7, thereby limiting nutrient and oxygen diffusion and increasing heterogeneity, which could compromise downstream analyses. Consequently, we restricted our observation to a 7-day period, which limits our observation window to 7 days. After confirming spheroid growth up to day 4, we tested both ONS and DAOY cells in their DTR and GFP-transfected conditions, enabling the development of a fully optimised 3D spheroid model for subsequent experiments testing the DTR/DT system.

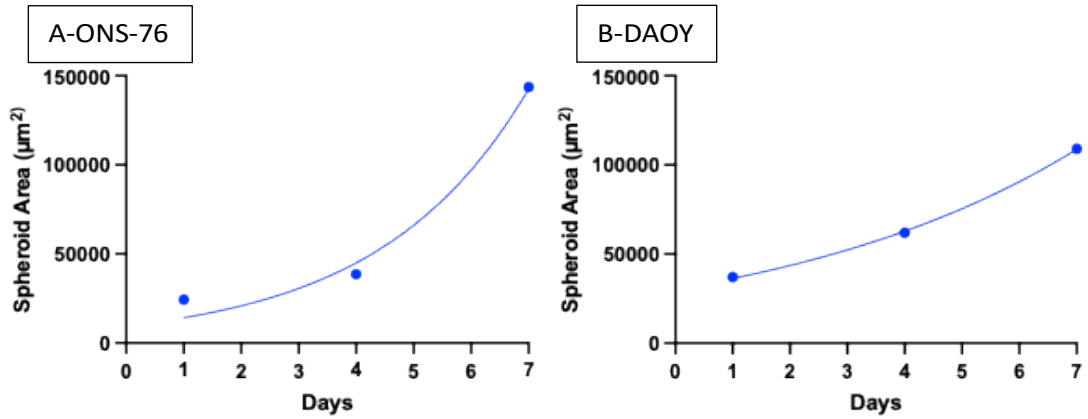


Figure 5 shows the spheroid growth of ONS-76 (3A) and DAOY (3B), quantified over a 7-day period and modelled using an exponential Malthusian growth equation based on a single data measurement. For ONS-76 spheroids, the best fit indicated an R^2 value of 0.9829, demonstrating a strong and stable growth phase over time. In contrast, DAOY spheroids had an R^2 value of 0.9995, reflecting a highly consistent growth trend during the same period. Data points were collected at three intervals: Day 1, Day 4, and Day 7. The fitted curves illustrate the growth dynamics of each cell line. ONS spheroids showed a higher proliferation rate, with a doubling time of 1.8 days, compared to DAOY, which had a doubling time of 3.79 days.

3.5 Comparative Analysis of the Impact of DTR Transfection on Basal Growth in 2D Models

The development of a reliable and reproducible genetically modified cellular model is a crucial step in preclinical research, especially when evaluating responses to therapeutic approaches. Lentiviral vectors are often used to facilitate stable gene delivery; however, these systems must undergo rigorous validation before testing to ensure they do not alter basal cellular growth rates or morphology (Lee et al., 2003).

Initial experiments were conducted using 2D monolayer cultures grown in T-75 flasks, a common approach for cell culture (Kapałczyńska et al., 2016). The percentage viability of cells over time served as a surrogate measure for growth rate, with comparative analyses between DTR-expressing cells and wild type assessed through an unpaired t-test. In the ONS-76 DTR-expressing models, analysis did not reveal a significant difference in proliferation, indicating that within the context of ONS-76 cells in 2D models, lentiviral transduction and DTR expression had a minimal effect on basal growth characteristics. Conversely, and unexpectedly, DAOY cells exhibited a statistically significant reduction in growth following DTR transfection, highlighting that DTR expression can alter growth kinetics independent of DT exposure. This effect may partly be due to the HB-EGF sequence present within the lentiviral vector, which could lead to perturbations in the EGFR-related signalling pathways, thereby affecting proliferation (Mitamura et al., 1995). This observation also aligns with previous evidence indicating that DAOY cells are particularly fragile concerning susceptibility to genetic or environmental stressors, attributable to their altered p53 signalling pathway (Castellino et al., 2007).

The analysis of the magnitude of differences, shown in Figure 7A, revealed that ONS-76 DTR-expressing cells exhibited relative fluorescence units (RFU) approximately three times higher than those of the wild type over the 7-day study period (2,831 ± SD vs. 894.1 ± SD). Although this difference was not statistically significant, it narrowly missed the p-value threshold of $p<0.05$ (calculated p-value was 0.0524), indicating a subtle biological shift in growth activity following the transfection of the DTR construct in ONS-76 cells.

Conversely, DAOY cells, as shown in Figure 7B, demonstrated an almost fourfold increase in RFU post-DTR transfection (2,703 ± SEM vs. 676.8 ± SD), with this difference reaching statistical significance ($p = 0.0421$). This highlights pronounced alterations in basal growth, suggesting heightened sensitivity compared to ONS-76 cell lines. These findings underscore both the differential vulnerabilities and metabolic responses of ONS-76 and DAOY cell lines to genetic manipulations.

In addition to the metabolic analyses, the stability of DTR construct expression was confirmed using fluorescence microscopy, as shown in Figure 8. Both ONS-76 and DAOY

cells exhibited strong GFP fluorescence in the DTR-GFP and GFP-transfected conditions, confirming the successful expression of the lentiviral construct. Whilst wild-type cells showed no GFP fluorescence, this further confirms the specificity of the model.

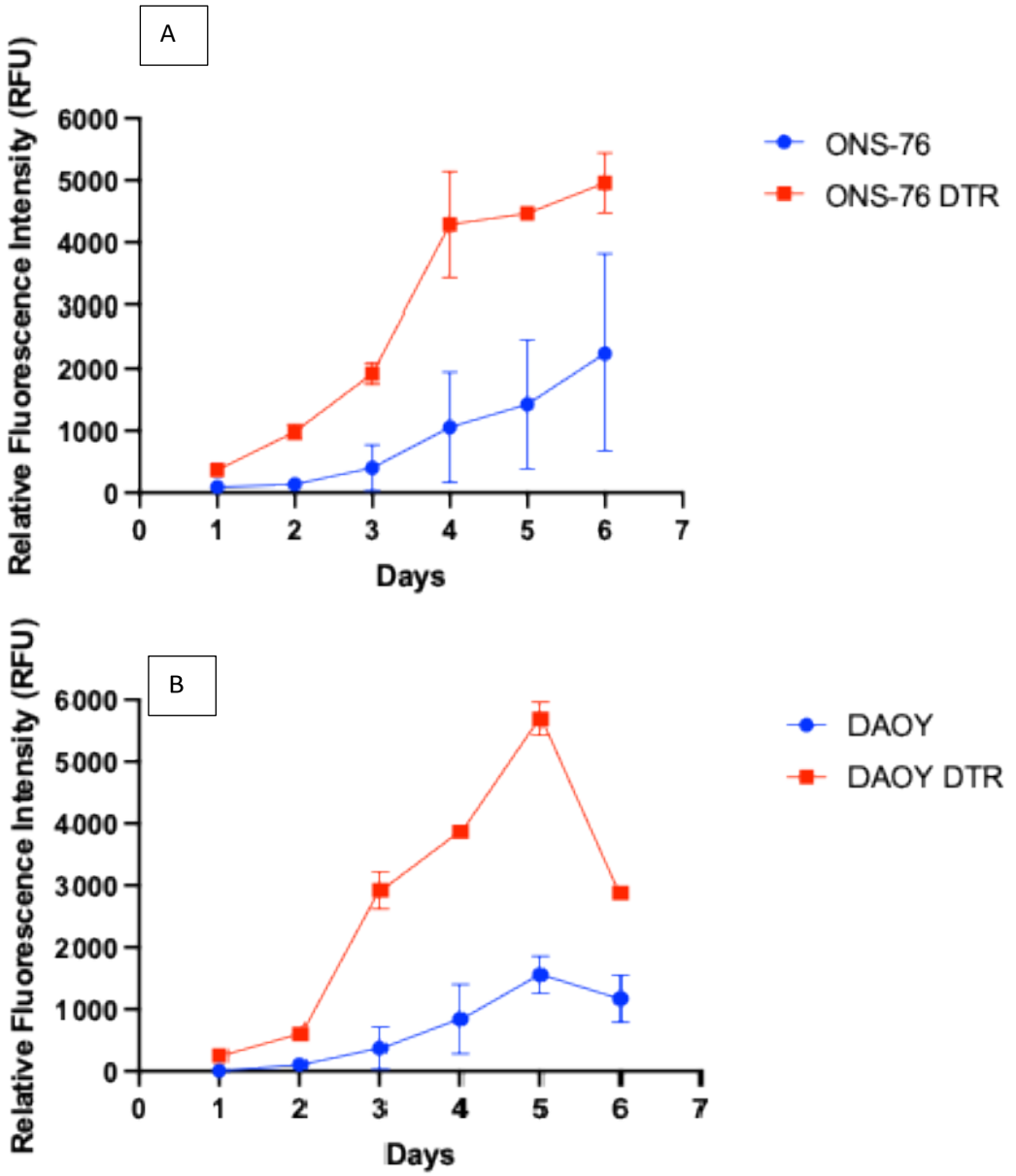


Figure 7-The metabolic activity of ONS-76 (7A) and DAOY (7B), cultured as 2D monolayers in 96-well plates, was quantified over six days using PrestoBlue cell viability assay to evaluate metabolic activity and proliferation. Daily measurements of Relative Fluorescence Intensity (RFU) were compared between wild-type and DTR-GFP-expressing populations. In ONS cells, DTR-GFP transfection resulted in higher RFU values compared to the wild type (2,831 ± SD vs. 894.1 ± SD). This difference was not statistically significant, as shown by an unpaired two-tailed t-test (t(10) = 2.200, p = 0.0524). Conversely, DAOY DTR-GFP cells exhibited significantly higher RFU than their wild-type controls (2,703 ± SEM vs. 676.8 ± SD), with the difference reaching statistical significance (t(10) = 2.329, p = 0.0421), indicating increased metabolic activity in DTR-GFP-expressing cells. Data is presented as mean ± SD, based on n=6 independent wells per group. A p-value of less than 0.05 was considered statistically significant. These results underscore cell line-specific differences in metabolic activity induced by DTR transfection, as measured by the PrestoBlue assay.

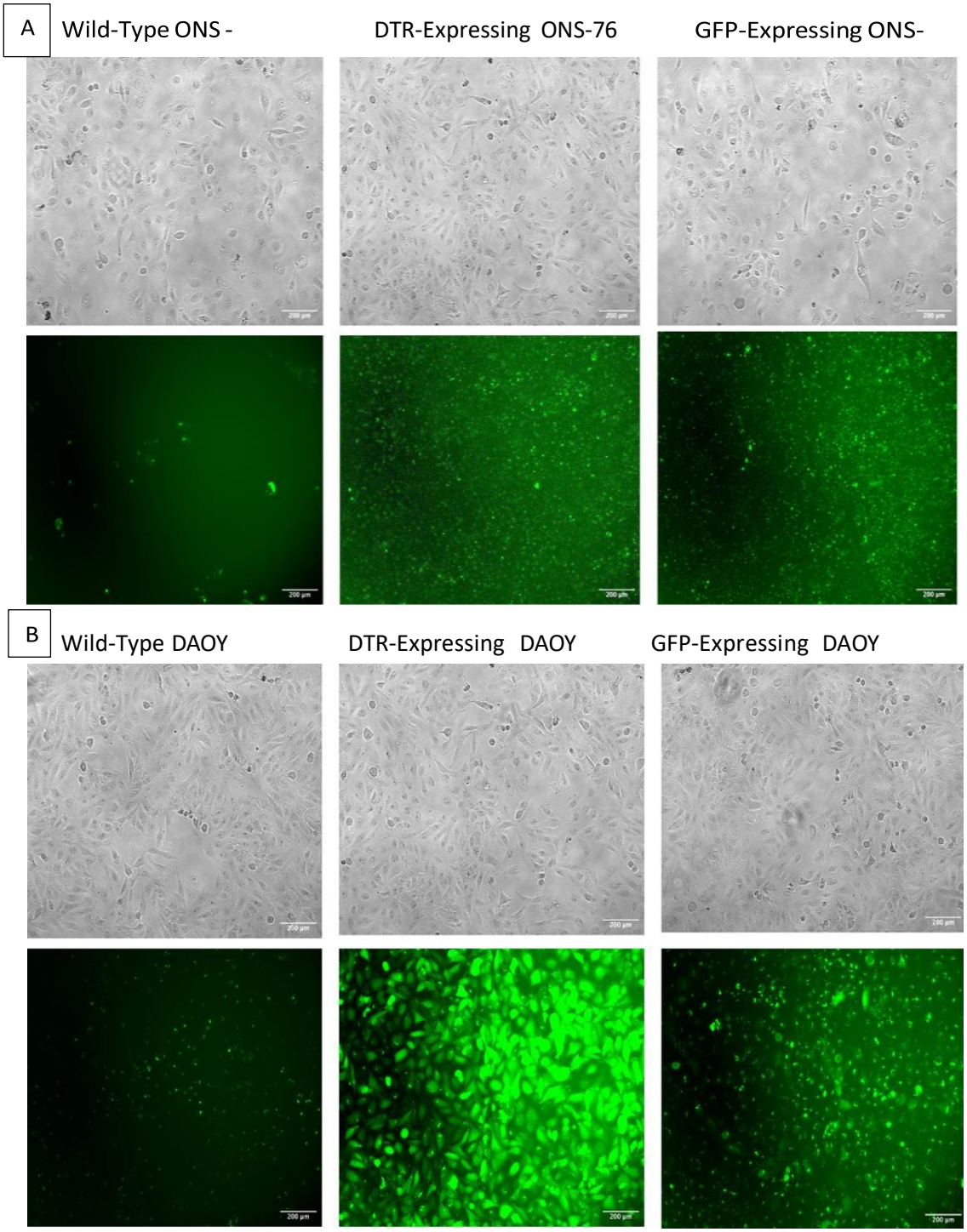


Figure 8 illustrates representative microscopy images of ONS-76 (A) and DAOY(B) cell lines as two-dimensional monolayers under three experimental conditions: wild type (Column A), DTR-GFP-expressing (Column B), and GFP-expressing (Column C). Images were acquired on Day 4 post-transfection to permit specific expression of the constructs. The images in Rows 1 and 3 correspond to brightfield microscopy, providing detailed information on cell confluency after four days of growth. The corresponding images in Rows 2 and 4 display GFP fluorescence captured under identical exposure settings across all images, facilitating direct comparison of GFP expression levels across conditions. The fluorescence panels confirm successful and stable expression of the DTR and GFP constructs in both ONS and DAOY cell lines, as evidenced by a strong, bright green fluorescence signal in the respective experimental groups. Conversely, wild type cells exhibited minimal GFP fluorescence, serving as a negative control for transfection specificity. The combined images substantiate the efficiency and specificity of the transfection procedures employed. Scale bars represent 200 µm.

3.6 Comparative Analysis of the Impact of DTR Transfection on Basal Growth in 3D Models

Three-dimensional models were employed to simulate more physiologically relevant conditions (Roper & Coyle, 2022). Both ONS-76 and DAOY cells were cultivated as spheroids over a 7-day period, with imaging performed on Days 1, 4, and 7 using the EVOS FL Auto System, as shown in Figures 10 and 11. Spheroid areas were quantified through a semi-automated macro on ImageJ, following the methodology described by Ivanov et al. (2016). Statistical evaluation involved one-way ANOVA and Tukey's post hoc analysis.

A significant observation was that, unlike in two-dimensional models, spheroid growth rates did not differ significantly among wild-type, GFP-transfected, or DTR-GFP-transfected conditions in either cell line. All experimental groups successfully formed viable and consistently proliferating spheroids, with no morphological alterations indicative of responses to altered proliferative capacity or cellular stress (Han et al., 2021). This absence of effect in three-dimensional culture conditions suggests that models with a more relevant representation of the tumour microenvironment may buffer potential perturbations resulting from viral integration or transgene expression.

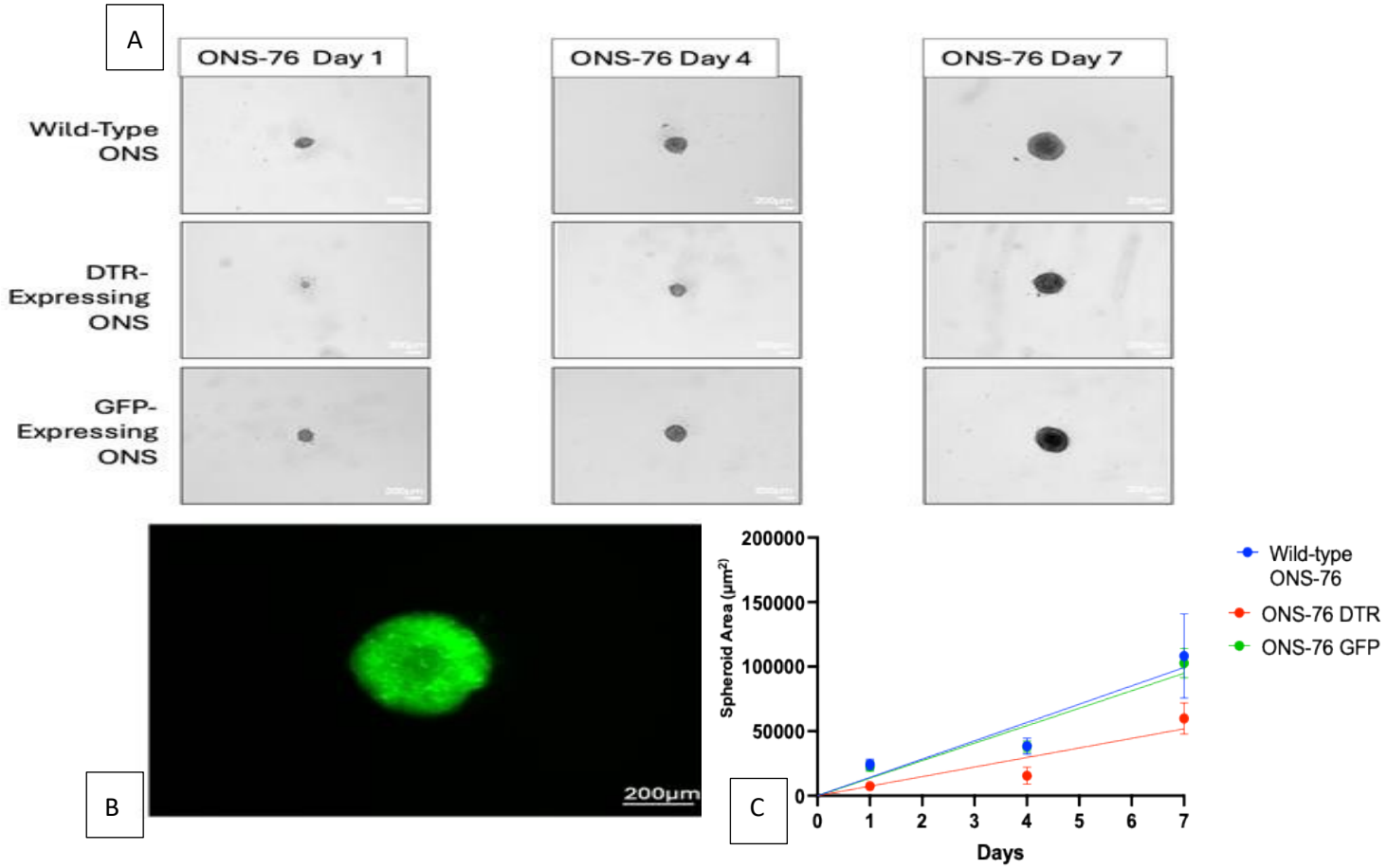


Figure 10- (A) Representative brightfield images of ONS-76 Spheroids cultured under 3D conditions in ULA 96-well plates. Cells were grown over a 7-day period, with images taken on days 1, 1,4, and 7 using the EVOS FL Auto System. Columns represent time points, while rows correspond to experimental groups: Wild Type (Row A), DTR-GFP-Expressing (Row B), GFP-transfected control (Row C). Over the 7-day period, all spheroids show progressive, controlled growth in morphology and size. (B) Fluorescent Imaging of GFP Reporter in DTR-GFP-Expressing ONS-76 Spheroids. (C) Quantitative analysis of spheroid area across the three groups, with statistical analysis using the Kruskal-Wallis Test (P=0.0396), indicating a significant difference in spheroid size among the groups. Dunn's post hoc multiple comparison shows significantly reduced growth in DTR-GFP-expressing ONS cells compared to wild type and GFP controls, consistent with impaired spheroid growth under DTR expression. Data are presented as Mean ± SD for n = 18 spheroids. Scale bars = 200 µm.

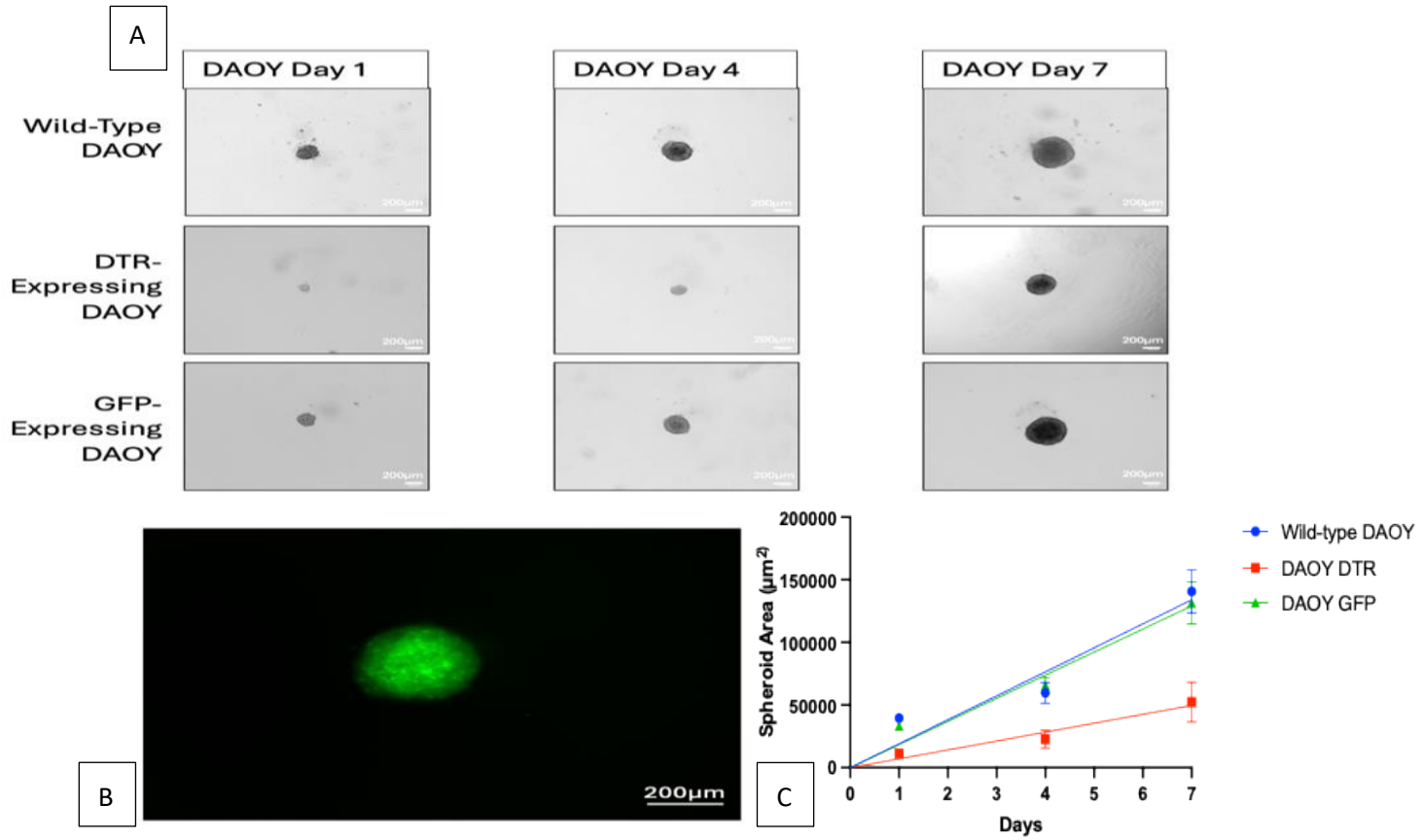


Figure 11-(A) Representative brightfield images of DAOY spheroids cultured under 3D conditions in ULA 96-well plates. DAOY cells were cultured over a 7-day period with images taken at Days 1, 4, and 7 using the EVOS FL Auto System. Columns represent time points, while rows correspond to experimental groups: Wild Type (Row A), DTR-GFP-Expressing (Row B), GFP-transfected control (Row C). Over the 7-day period, all spheroids exhibited progressive, controlled growth in morphology and size, with no statistically significant differences between groups at Day 7. (B) fluorescent imaging of GFP as a market for DTR-GFP-expressing DAOY cells. (C)Quantification of spheroid area was performed using a semi-automated ImageJ Macro, with a further one-way ANOVA revealing no statistical significance in mean spheroid area between the three groups ($F(2,15) = 2.087$, $P = 0.1587$). Furthermore, post hoc Tukey's multiple comparisons further confirmed the absence of statistical differences between DTR-GFP, GFP, and wild-type spheroids. Data represent mean ± SD for $n = 18$ spheroids. Scale bars = 200 µm.

3.7 Differential Sensitivity of ONS-76 and DAOY to DTR/DT-Mediated Ablation in 2D and 3D Cultures

The primary objective of this study was to evaluate the effectiveness of the DTR-GFP/DT Model in enabling the selective ablation of medulloblastoma cell populations. To establish this model, we generated 3D spheroids from FACS-sorted DTR-GFP, ONS-76 and DAOY cells, ensuring through GFP fluorescence that all cells uniformly expressed the DTR construct. GFP-expressing and wild-type cells were included as controls to distinguish the effects specifically of DTR-mediated DT sensitivity. The significance of this study lies in the model's ability to specifically target cells engineered to express the DTR, giving this approach potential for the experimental ablation of particular cell populations.

The effects of DT treatment were primarily assessed in 2D monolayer conditions on both ONS and DAOY cell lines. For the ONS 2D cultures, viability measurements were taken after 48 hours of incubation with a medium containing DT. Non-normally distributed data revealed significant differences in cell viability between the ONS-DTR, ONS-GFP, and wild-type ONS groups, indicating that DT treatment had an impact on cell viability. However, this result is somewhat counterintuitive, as additional pairwise post hoc comparisons did not reach statistical significance. Although a reduction in viability was observed in 2D conditions, it is not sufficient to definitively conclude the cytotoxic effects of DT in ONS 2D conditions, which may reflect variability in DTR expression or limited accessibility of DT in 2D monolayer conditions.

In parallel, the impact of DT exposure on our DAOY-derived cell lines was assessed under identical 2D conditions. Remarkably, unlike ONS-76-derived cell lines, DAOY showed no significant decrease in cell viability across all treatment groups in 2D conditions, as confirmed by Tukey's post hoc analysis, which was based on the data meeting normality assumptions. This variation may be attribution to the inherent differences of DT uptake between cell lines, or underlying resistance mechanisms reinforcing the need to evaluate cytotoxicity in 3D spheroid cultures on a cell-line specific basis.

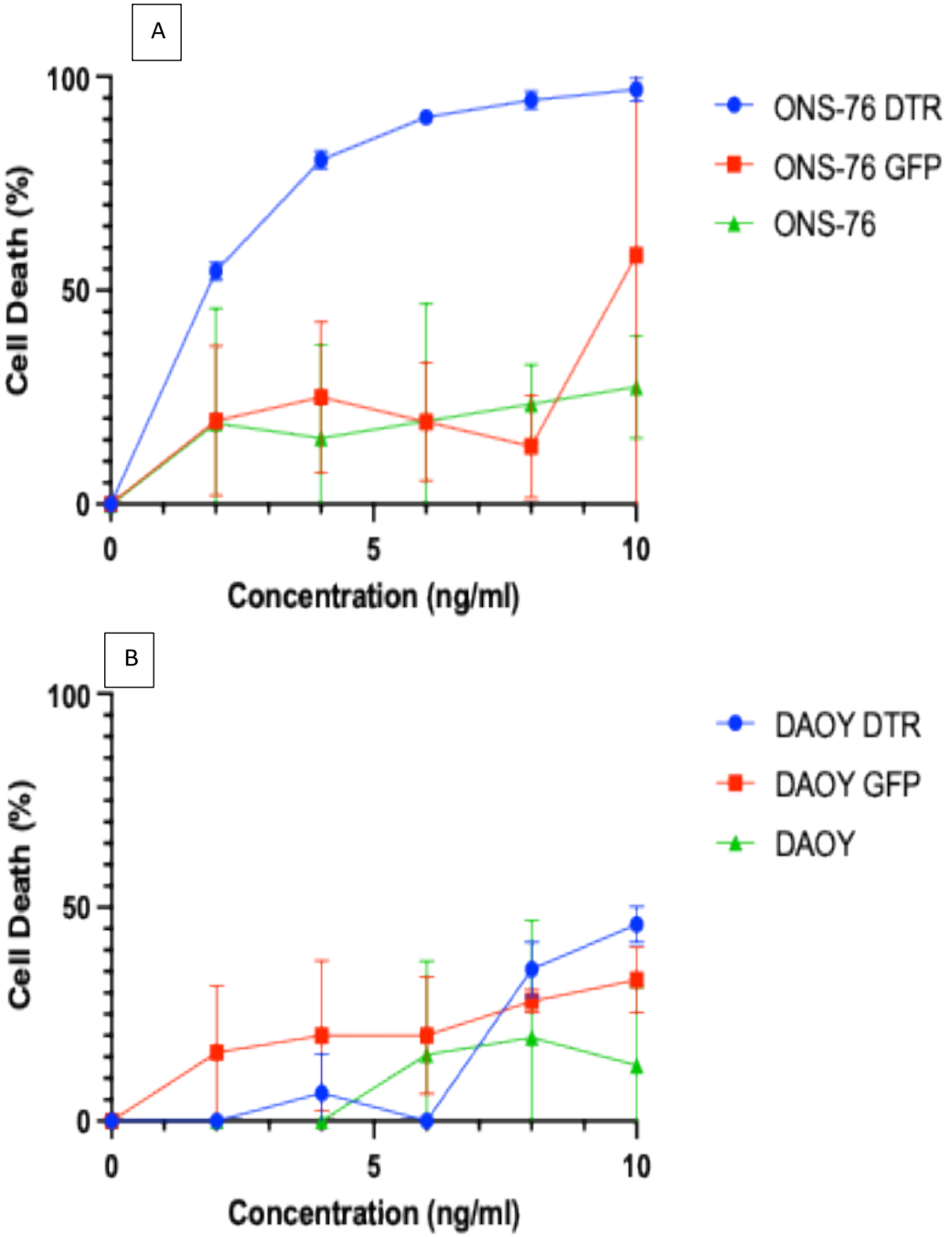


Figure 9 (A) shows the percentage of cell death observed following DT treatment in ONS-76 medulloblastoma cells cultured in 2D under three experimental conditions: wild-type, DTR-GFP-expressing, and GFP-transfected controls. Each group consists of n = 6 replicates, with results presented as the Mean ± SD. The Kruskal-Wallis test revealed no statistically significant difference in cell death percentage across the three groups (Kruskal–Wallis statistic = 5.105, P = 0.0727), and further multiple comparisons using Dunn's Post Hoc test confirmed the absence of significant pairwise differences, with mean rank differences: DTR vs GFP = 5.750; DTR-GFP vs wild-type = 6.250; GFP vs wild-type = 0.5000. This suggests a slight, but not statistically significant, tendency towards increased cell death in DTR-GFP-expressing cells. (B) Depicts the percentage of cell death in DAOY cells following DT treatment under identical experimental conditions. Data represent mean ± SD from n=6 biological replicates per group. One-way ANOVA indicated no statistically significant differences in mean cell death among wild-type, DTR-GFP-expressing, and GFP-transfected DAOY cells ($F(2,15) = 0.9434$, $P = 0.4112$; $R^2 = 0.1117$). Further post hoc Tukey's analysis confirmed that there were no significant pairwise differences between groups. Overall, these results demonstrate that, under 2D conditions, neither cell line exhibits a marked response to DT treatment.

3.8 Diphtheria Toxin Selectively Reduces Viability in DTR-Expressing ONS and DAOY Cells in 3D models

Subsequent experiments were conducted to assess the effect of DT on DAOY- and ONS-76-derived 3D culture systems, using the same experimental conditions. In ONS-76 spheroids with DTR-GFP expression, a notable and significant vulnerability to the cytotoxic effects of DT was exhibited. One-way ANOVA with Šídák's post hoc analysis showed a highly significant, dose-dependent reduction in cell viability in DTR-GFP-expressing spheroids only, compared to both wild-type and GFP controls, as seen in Figure 12a. Significant differences were observed with a small dose of 2 ng/ml of DT, whereas a high dose of 10 ng/ml caused DTR-GFP-expressing ONS-76 spheroids to exhibit near-complete ablation. At the same time, both GFP-transfected and wild-type groups remained relatively unaffected. These results underscore the remarkable specificity and efficacy of DT as a cytotoxic agent in the DTR/DT model.

Furthermore, in DAOY spheroids, a similar trend regarding dose-dependent effects of DT was observed, although this was slightly delayed compared to ONS-76 spheroids. No significant difference was evident between experimental groups until 12 ng/mL of DT, with the effect size increasing at higher doses of 16 ng/mL and 20 ng/mL. At these higher concentrations, DTR-GFP-expressing spheroids showed a striking reduction in viability compared to GFP controls and wild types, which exhibited little effect from DT treatment.

Together, these findings confirm the specificity of the DTR/DT model for use in ONS-76 and DAOY medulloblastoma spheroid models. However, it is important to note that DAOY spheroids were notably less sensitive to the cytotoxic effects of DT than ONS-76 spheroids, reflecting cell-line-specific differences within this model.

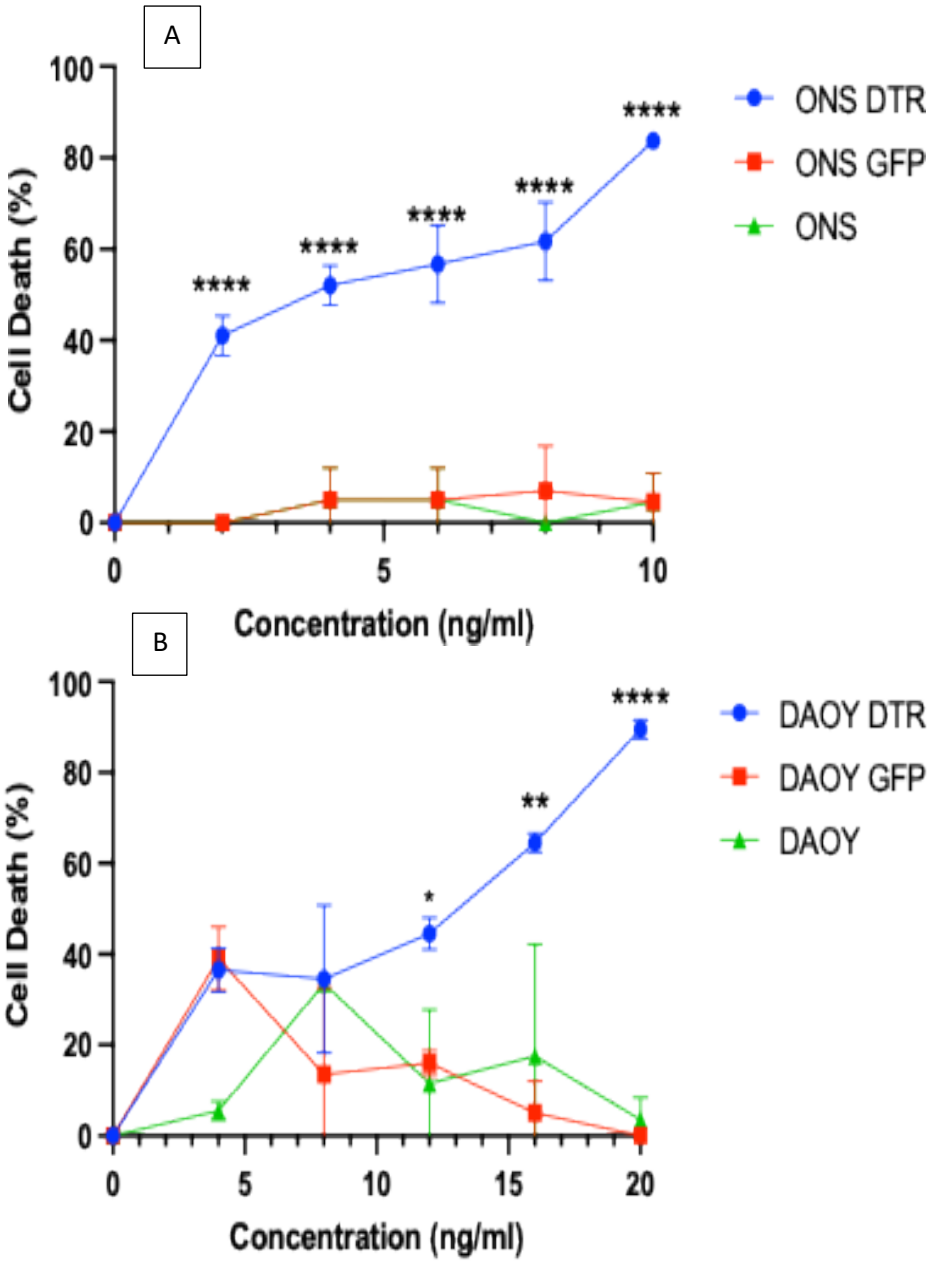


Figure 12 (A) shows the percentage of cell death in ONS-76 spheroids grown in a ULA 96-well plate, measured by Titer-Glo assay after DT treatment under three experimental conditions: wild-type, DTR-GFP-expressing, and GFP-transfected controls. Data are presented as mean ± SD (n = 18 spheroids per group). One-way ANOVA revealed a significant difference between the experimental groups ($F(2,15) = 16.10$, $P = 0.0002$, $R^2 = 0.6823$), with further multiple comparisons using Šídák's multiple comparisons confirmed significant greater DT-induced reductions in cell viability in only the DTR-GFP-expressing spheroids compared to GFP transduced (mean difference = 45.58, 95% CI = 21.18–69.99, P = 0.0006) and wild-type spheroids (mean difference = 46.75, 95% CI = 22.35–71.15, P = 0.0005). Significant differences emerge from >2ng/mL. (B) Cell death was assessed in DAOY spheroids following DT treatment under the same experimental conditions. The use of Šídák's multiple comparisons identified significant differences in reduction in cell viability following DT treatment in DTR-GFP-expressing spheroids at 12 ng/ml (P = 0.0391), 16 ng/ml (P = 0.0033), and 20 ng/ml (P < 0.0001) compared to controls. Both wild-type and GFP-transduced DAOY spheroids remained resistant to DT treatment.

4.0 Discussion

This study aimed to assess and evaluate the efficacy and specificity of the DTR/DT model system in two distinct medulloblastoma cell lines, ONS-76 and DAOY, under both 2D and more physiologically relevant 3D conditions. The study further sought to determine the effective conditions for antibiotic selection while assessing whether DTR transfection itself affected cellular growth dynamics.

Upon reviewing the literature, no studies were found that specifically assessed the DTR/DT system in medulloblastoma models. This study is therefore the first of its kind to successfully demonstrate selective and reproducible DTR-mediated medulloblastoma ablation within a physiologically relevant three-dimensional (3D) spheroid system. This innovative model not only offers a more accurate representation of tumour biology but also marks a significant advancement over 2D culture models, opening new avenues for the long-term study of survivorship.

4.1 Puromycin Sensitivity and Implications for Stable Clone Generation

The generation of stable cell populations was a crucial prerequisite for optimising downstream experiments, ensuring only successfully transduced cells were used. Determining the optimal sensitivity across both ONS-76 and DAOY cell lines ensured efficient establishment of a procedure for stable clone generation and upheld the validity of downstream conclusions.

4.1.1 Rationale for Puromycin Selection

Puromycin is a widely employed antibiotic for the development of stable transfected populations due to its potent cytotoxic effects, even at low concentrations (Miyata et al., 2022). Establishing cell line-specific sensitivities for ONS-76 and DAOY cells was an essential first step to ensure efficient generation of populations with only transfected cells, whilst maintaining cell viability.

4.1.2 Dose Dependent Cytotoxicity of ONS-76 and DAOY Cells

The initial findings of this study confirmed the use of puromycin as an effective means of induced cytotoxicity in a dose-dependent manner in both wild-type ONS-76 and DAOY cells, as shown by both cell lines exhibiting significant negative correlations between puromycin concentration and percentage cell viability, supporting previous studies that highlight the cytotoxicity of puromycin It is somewhat surprising to note that DAOY cells required a higher concentration of puromycin to achieve complete cell death compared to ONS-76 cells-These findings corroborate previous research indicating that DAOY cells' greater drug efflux is due to higher expression of ABC transporters Consequently, a higher dose is needed to see a cytotoxic effect.

4.1.3 Application for Downstream Use of Transfected Populations

These findings were utilised post-lentiviral transduction with GFP-expressing clones to generate a population of only transduced cells. Inspection of morphological characteristics indicated that puromycin selection did not cause an overt cytotoxic effect in the surviving populations beyond its intended effect.

4.1.4 Implications for Stable Clone Generation

These findings were critical for confirming the feasibility of generating stably transfected ONS-76 and DAOY cell populations, while also underscoring the need to tailor antibiotic selection protocols for different cell lines to ensure optimal effectiveness. Moreover, this data provides a reassuring foundation for developing a reliable method to select transfected ONS-76 and DAOY cell lines, emphasising the need for cell-line-specific optimisation procedures.

4.2 Optimisation of DTR/DT Transfection Model Using ONS-76 and DAOY Cells as a Baseline

The optimisation of a system for DTR/DT transfection was a crucial step to ensure the reliability and reproducibility of our downstream experiments. Key parameters, including MOI, polybrene, and spin inoculation, were systematically tested to ensure maximum transduction effects while minimising any cytotoxic effects. An MOI of 1, combined with polybrene and spin inoculation, yielded optimal conditions for growth. This optimisation step provided a robust baseline for specific ablation experiments, ensuring stable expression of DTR without any confounding cytotoxic effects.

4.2.1 Rationale for Cell Line Selection

The use of ONS-76 and DAOY cells provides a system for refining transduction conditions by assessing the effectiveness of construct transfection through monitoring GFP fluorescence under a fluorescence microscope. These subtypes of Shh medulloblastomas are commonly recognised as resilient cell lines with high proliferative and migration potential (Bonfim-Silva et al., 2019), making them ideal for use in our study.

4.2.2 Key Parameters to Enhance Transduction Efficiency

To determine the optimal conditions for maximising DTR gene delivery and efficiency,

combinations of three variables that influence lentiviral transduction efficiency were tested: multiplicity of infection (MOI) (Zhang et al., 2004), polybrene use (Han et al., 2015), and spin inoculation (Guo et al., 2011). Polybrene is a cationic polymer that acts by neutralising negative charges within the viral construct and the cell surface, thereby reducing electrostatic repulsion, which enhances the binding and entry of the virus into the target cell, consequently increasing the efficiency of viral transduction (Davis et al., 2002). Spin inoculation further promotes transduction efficiency by facilitating close contact between viral particles and target cells, thereby increasing the likelihood of successful transduction (Dodo et al., 2014).

4.2.3 Optimisation of Transfection Protocols

It was observed that increasing the MOI beyond 1 did not correlate with higher GFP expression levels when tested independently without polybrene or spin inoculation, aligning with findings previously reported by Cockrell and Kafri (2007). The study indicated that an excessively high MOI could lead to cellular toxicity and a saturation plateau, which would dull transduction efficiency (Cockrell & Kafri, 2007). Consequently, utilising an MOI of 1 represents a compromise between the high transduction efficiency and minimising cytotoxic effects associated with higher MOI values.

4.2.4 Reproducibility and Validation

This optimisation step was essential in our experiment, as it improved the reproducibility and consistency of our results on ONS-76 and DAOY cells. Establishing a reliable model for DTR transfection lays a foundation for future research using the DTR/DT model to target specific cell populations.

4.2.5 Implications for Future Research

Overall, the successful establishment of a DTR/DT transfection model provides a valuable tool for the selective ablation of cells in medulloblastoma research. The refinement of transduction conditions ensures that subsequent functional assays can utilise this to improve reproducibility.

4.3 Effects of DTR Transfection on Basal Cellular Growth

Assessment of basal growth following DT transfection was a crucial step in distinguishing the effects of DTR transfection from the cytotoxic effects induced by DT treatment, ensuring that any observed cell death could be attributed to the toxin's action rather than transfection effects. ONS-76 cells maintained comparable effects across both 2D and 3D models, with no significant impact of transfection on growth. By contrast, DAOY cells exhibited significantly reduced proliferation in 2D models, yet this

was not observed in 3D conditions, suggesting that dimensionality may buffer the inhibitory effects of transfection.

These results emphasise the necessity of validating the effects of genetic modifications across cell lines to ensure the validity of downstream experiments. Collectively, these findings support the robustness of the DTR/DT system, particularly in ONS-76 cells, providing a reliable framework for future studies involving ablation.

4.3.1 Rationale for Assessing Basal Growth

A crucial objective of the project was to assess whether the DTR construct transfection influenced baseline growth characteristics in ONS-76 and DAOY cell lines. This aspect of our investigation was essential for distinguishing the effects of the DTR construct from those resulting from DT treatment, ensuring the reliability of any downstream analyses performed. Upon reviewing the literature, data from several studies suggest that the transfection of viral constructs can impact cellular growth, either through reduced cell viability or increased growth (Kim & Eberwine, 2010; Sharma et al, 2018). Therefore, it is crucial to investigate whether the DTR construct affects cellular proliferation before examining toxin-induced cell death.

4.3.2 Effects of Transfection in 2D Monolayer Models

In the case of ONS-76 cells post-transfection, we observed no significant differences in growth rates in 2D conditions between wild-type counterparts. While it should be noted that the p-value was close to significance ($p = 0.05224$), this difference was not considered statistically significant; therefore, we can conclude that the presence of the DTR construct in our 2D cultures does not appear to be detrimental to the viability of these cells. These findings support the use of the DTR system in ONS-76 cells for subsequent experiments to assess the cytotoxic effects of DT.

In contrast, analysis revealed a significant reduction in DAOY cell growth following DTR-GFP transfection under 2D conditions ($p=0.0421$), suggesting that the DTR construct plays an inhibitory role in DAOY cell proliferation within 2D models. One possible explanation for this is that the reduced viability is attributable to the metabolic stress imposed by transfection on cells, which can ultimately lead to cell death and apoptosis (Kim et al., 2018). The mechanisms behind this process vary between cell types, and due to limited research on transfection in DAOY cells, the precise cause remains unclear; perhaps the upregulation of stress response pathways and increased protein synthesis contribute to this metabolic burden (Avci-Adali et al., 2014; Joutsen, 2018).

It is essential to note that the magnitude of the observed effects was relatively small; therefore, future research should include more biological replicates to evaluate the strength of these conclusions better. Nonetheless, this finding underscores the need to validate the physiological impact of transfection on a cell-by-cell line basis, rather than

assuming uniformity across cell lines.

4.3.3 Effects of Transfection in 3D Spheroid Models

To further explore the influence of DTR transfection on proliferation levels, the analysis was extended to 3D spheroid cultures, generating results that are more physiologically relevant, as the model provides a more accurate representation of tumour growth in vivo (Roper et al., 2021). In both ONS-76 and DAOY spheroids, no significant differences were observed between wild-type and transfected conditions. Therefore, these findings suggest that within 3D culture conditions, the DTR construct does not significantly affect basal growth dynamics, thus supporting previous work by Duval et al. (2017), which suggests that 3D spheroids, as opposed to 2D monolayers, dampen the growth changes that may be observed in 2D, possibly due to cell-cell and varied cell-matrix organisations.

The absence of pronounced impairments in growth following transfection in 3D cultures indicates that the increasingly complex architecture of 3D conditions may serve to buffer or mitigate any responses to transfection observed under 2D conditions. Specifically, in DAOY cells, we observe a reduction in proliferation in 2D environments. However, in 3D cultures, the growth rates observed in 3D seem to normalise. This normalisation may be explained by the generally slower proliferation rates observed in 3D conditions, as reported by Rybkowska et al. (2023), which could diminish or mask any growth defects directly caused by the transfection of the DTR-GFP gene.

4.3.4 Implications of Dimensional Context

These findings emphasise the importance of validating transfection modifications under both 2D and 3D conditions, rather than assuming uniformity between them. The fact that DAOY cells did not show growth in 3D, yet exhibited a significant depletion in 2D, is particularly relevant for the development of future models, as it indicates that observations in 2D conditions do not accurately reflect those seen in vivo.

Taken together, these results indicate the strength of the DTR/DT model as it does not significantly impact growth rates in ONS-76 in 2D or 3D conditions. However, it is important to note the discrepancy in that whilst a reduction in growth was observed in DAOY 2D, this effect was not observed in 3D conditions, suggesting this impact on growth rate is dependent on culture context, as 3D conditions better mimic in vivo conditions and alter cellular behaviour (Edmondson et al., 2014). These results are encouraging and support the use of the DTR/DT mode, particularly within ONS-76 cells, as a valid model for investigating the effects of DT-mediated cytotoxicity. Notably, the observed plateau of growth rate post day 7 defines a critical experimental window that future studies may utilise, forming the backbone of the development of a dimensionally accurate selective ablation system.

4.4 Dimensionality Influences Sensitivity to Diphtheria Toxin

This study demonstrated the crucial role of dimensionality in shaping the cytotoxic response of the DTR/DT system, where our 3D spheroid cultures showed increased sensitivity compared to 2D monolayer cultures. The greater efficacy observed in 3D models highlights the advantages of spheroid models. Consequently, using 3D spheroids in this study provides superior predictive power for translational applications, reinforcing the need for future studies to develop further the DTR/DT system for targeted ablation therapies.

4.4.1 Differences in Cytotoxicity Between 2D and 3D Models

One of the most significant findings in this study was the prominence of differences in cytotoxicity observed between 2D and 3D models, which may be attributed to the unique pharmacodynamic differences within the architecture of 3D spheroid models, affecting diffusion characteristics through the extracellular matrix (Langhans, 2018). This variation, compared to 2D models, may be responsible for the increased effect of DT due to the prolonged and localised exposure that 3D models experience, resulting from the confinement in spatial availability for diffusion, which increases the likelihood of toxin internalisation.

4.4.2 Limitations of 2D Monolayers in Drug Response Studies

The limitations of 2D models in drug responses are well studied. Several prior studies have demonstrated differences in drug resistance mechanisms between 3D cultures and 2D cultures (Breslin & O'Driscoll, 2013), particularly in terms of altered extracellular matrix structure, compact cell junctions, and hypoxic gradients that are uniquely present in spheroid cultures (Knight & Przyborski, 2014). In the context of our research, the compact environment of a spheroid architecture may have acted to prolong DT exposure due to slower diffusion rates within spheroid architecture (Tong et al., 2016), or the more physiologically relevant conditions which could enhance DT internalisation and thus increase cytotoxic ability (Juarez-Moreno et al., 2022). By contrast, the observed lack of significance of the effect of DT in 2D cultures resonated with research that limits the use of 2D cultures for drug studies due to their reduced predictivity and simplified interactions (Kapałczyńska et al., 2016; Mameishvili, 2025), which in our study led to a reduced probability of sustained interaction with DTR in 2D cultures.

4.4.3 Advantages and Disadvantages of 3D Spheroid Models

Whilst 3D cell cultures are widely considered to be more stable (Zingales et al., 2023) and physiologically relevant (Roper et al., 2021), they come with inherent disadvantages regarding toxin susceptibility (Leite et al., 2019). For example, research by Knight and Przyborski (2015) highlights the innate differences in drug sensitivity models, particularly increased resistance to chemotherapeutic agents in 3D cultures, which is attributed to spatial constraints that are not observed in flat monolayer cultures. Similarly, research by Breslin and O'Driscoll (2016) demonstrates that spheroids exhibit distinct differences in exposure to bioactive compounds, underscoring the importance of 3D models in capturing intratumour heterogeneity.

4.4.4 Divergent Findings Compared to Chemotherapeutic Responses

The findings of this study align with the work by Breslin & O'Driscoll (2013), which observed varying cytotoxicity of drugs when using more physiologically relevant 3D spheroids compared to 2D monolayers, in terms of increased drug penetration and retention, leading to increased interaction of the toxin with its respective receptor. However, these findings are contrary to previous studies that demonstrate increased resistance to chemotherapeutic drugs in 3D cultures (Edmondson et al., 2014). This divergence from the observed norm can be partially explained by the fundamental differences in the DTR/DT approach to standard chemotherapeutics.

4.4.5 Translational Significance of 3D Models

Taken together, the enhanced response of ONS-76 Spheroids to the DTR/DT model system highlights the importance of using biologically relevant in vitro models when evaluating drug efficacy. This necessity arises due to the limitations of conventional 2D cell culture models, which often fail to accurately mimic the complex biological environments found within the human body, resulting in reduced efficacy and specificity. By integrating structural considerations into the model design, including three-dimensional architecture, extracellular matrix components, and cell-cell interactions, the research derived from this approach will provide a much more robust and reliable platform, facilitating significant advancements in translational research and therapeutic development. For example, medulloblastomas in vivo within dense, ECM-rich architectural conditions that profoundly influence tumour behaviour and drug response (Orr, 2020). These observations in 3D cultures provide a more accurate reflection of in situ physiological interactions and pharmacodynamics, closely mimicking the tumour microenvironment.

Additionally, the 3D model developed in this study, which better replicates spatial and molecular constraints, aligns with findings from recent studies such as Mangani et al. (2025) and provides superior predictive power, making it a reliable system for designing, testing and optimising DTR/DT-based ablation therapies. Ultimately, adopting such

complex, physiologically relevant models is essential for bridging the gap between in vitro experiments and clinical applications, ensuring that therapeutic strategies are both practical and targeted.

4.5 Functional Efficacy of the DTR/DT System in ONS-76 Cells

ONS-76 cells were highly responsive to the DTR/DT model system, showing both statistically significant robustness and elevated levels of cytotoxicity in response to treatment. The rapid and exclusive effects observed in 3D models confirm the specificity of the DTR/DT model and highlight its physiological relevance to the tumour microenvironment. These findings establish ONS-76 as a benchmark for the use of a targeted ablation model for Shh medulloblastomas, against which the response of other cell lines can be compared.

4.5.1 Statistical Outcomes and Observed Limitations

The most striking result in this study, ONS-76, a Shh medulloblastoma subtype, exhibited a marked sensitivity to the cytotoxic effects of DT following DTR-GFP transduction. The most pronounced cytotoxic response was observed in 3D spheroid cultures, where a rapid and significant reduction in cell viability occurred within 48 hours of DT administration. Furthermore, this cytotoxic effect was highly specific to transfected populations, with our GFP controls and untransduced wild-type ONS-76 spheroids not showing significant reductions in cell viability. Hence, these findings align with our expected cytotoxic effects of DT solely in DTR-GFP transfected populations due to its specific actions by the presence of its receptor on the cell membrane (van Blijswijk et al., 2014).

4.5.2 Biological Significance of the Response

An additional significant aspect of this study is the observation that the highly potent cytotoxic effect of DT transpires within a relatively brief exposure period, specifically within 48 hours. This finding highlights the efficacy of the model and its capacity to evoke a significant and robust biological response expeditiously. Such outcomes are particularly noteworthy, as they have important implications for the development of innovative therapeutic strategies that selectively target specific effects without compromising overall integrity.

4.5.3 Implications for Future Studies

The significance of this research lies in its capacity to advance personalised medicine and targeted therapies, thereby facilitating effective treatment outcomes with fewer adverse effects, as suggested by Brockschnieder et al. (2006). These findings also

underscore the need for longitudinal evaluation of the cytotoxic effects of DT, extending beyond the initial 48-hour period observed. While the current study demonstrates a rapid and notable level of cytotoxicity in ONS-76 cell lines, further research is needed to understand the effects of cell death beyond mere population ablation. Such investigations are essential to allow for the development of models that incorporate long-term study periods, thereby elucidating neurodevelopmental and cognitive effects resulting from the utilisation of this model. These studies will be vital in determining whether the cytotoxic effects of the model emulate long-term consequences seen in tumour ablation, which hold greater physiological relevance in the context of therapeutic applications.

4.5.4 Mechanistic Explanations for Enhanced Efficacy

It is challenging to explain these results due to the scarcity of comprehensive literature on the subject; however, a plausible explanation for the striking cytotoxicity observed in 3D models arises from research conducted by Edmondson et al. (2014). This research suggests that 3D models are more structurally relevant due to their ability to preserve spatial architecture and maintain physiologically pertinent oxygen gradients.

4.5.5 Impact of 3D Architecture on Cytotoxic Responses

One interpretation of the findings is that three-dimensional models enhance receptor clustering on the cell surface, thereby facilitating receptor-ligand interactions and promoting increased cellular uptake through endocytosis (Maurer & Cooper, 2005). In this specific model, clustering of DTR on the cell surface amplifies the uptake of diphtheria toxin (DT), thereby intensifying the downstream cytotoxic response, which explains the prominent reduction in cell viability observed in the study (Maurer & Cooper, 2005). This observation is particularly relevant due to its physiologically relevant environment; hence, it supports the physiologically superior nature of three-dimensional models, while also increasing the models' significance within clinically relevant developments.

In summary, ONS-76 cells represent a highly responsive model for the DTR/DT system, exhibiting both statistical robustness and biological potency in their response to toxin administration. The rapid and specific cytotoxic effects observed in three-dimensional spheroid cultures validate this system as a reliable and physiologically relevant platform for investigating receptor-targeted therapies. Beyond the striking and profound cytotoxic responses observed in this study, this model highlights how variations in cell lines can determine therapeutic effectiveness, offering a valuable contrast to the observed slight resistance in DAOY-derived cell lines. By considering ONS-76 cells as a more sensitive cell line for DT treatment, this can inform experimental design and enhance the translational applicability of the DTR/DT model.

4.6 Differential Efficacy of the DTR/DT system in DAOY cells

In contrast to the stark, significant cytotoxicity observed in the ONS-76 models, DAOY lines exhibited a more gradual and delayed response following DT exposure. While DAOY-DTR-GFP expressing spheroids did not exhibit sensitivity at the same concentrations as ONS-76 DTR-GFP expressing spheroids, significant reductions were observed from 12 mg/mL of DT onwards, with more pronounced significant effects at 16 ng/mL and 20 ng/mL, achieving almost complete cell ablation. These findings highlight the differences between SHH cell lines, with DAOY cells being less sensitive to the cytotoxic effects of DT, yet still susceptible to DTR/DT-mediated cytotoxicity at high concentrations.

The following subsections examine the statistical outcomes, biological relevance, and potential explanations for the reduced efficacy of the DTR/DT model in relation to DAOY.

4.6.1 Statistical Outcomes and Observed Limitations

In contrast to the pronounced and significant cytotoxic effects observed in ONS-76 cells at low concentrations, DAOY-derived cell lines exhibited a considerably more gradual dose-response to DT treatment, with this response notably increasing at higher concentrations. One-Way ANOVA with Šídák's post hoc analysis confirmed that DAOY DTR-expressing spheroids were sensitive to the cytotoxic effects of DT at much higher concentrations than ONS-76 spheroids, with significant reductions in viability appearing at concentrations exceeding 12mg/ml. These findings demonstrate the effectiveness and specificity of the DTR/DT model in DAOY spheroids; importantly, they elucidate that a higher sensitivity threshold is required when compared to ONS-76. This increased sensitivity highlights the variability between SHH-derived medulloblastoma cell lines, thereby underscoring the need to consider cell-line-specific differences when using the DTR/DT model.

4.6.2 Biological Significance of DAOY Results

It is of biological and statistical importance to note the differential sensitivity to the cytotoxic effects of DT observed in our DAOY spheroids. DTR-GFP-expressing DAOY spheroids exhibited significant reductions in cell viability once the threshold dose, established to be around 12ng/mL of DDT, was reached. In contrast, the effects of DT across wild-type and GFP controls remained nearly undetectable and resistant at all doses.

The observed variability between ONS-76 and DAOY responses underscores the importance of considering both biological and statistical significance when evaluating the DTR/DT model for cell-specific medulloblastoma ablation models. The absence of a significant response at low doses does not equate to resistance to DT treatment but

rather reflects a right-shifted sensitivity curve. The ability of DAOY spheroids to undergo significant levels of cell death, even at higher concentrations, reinforces the capacity of the DTR/DT model for DAOY cell lines, albeit with cell-line-specific constraints and variabilities.

4.6.3 Implications for Future Studies

The necessity for a higher dose to observe cytotoxicity in DAOY spheroids necessitates further research aimed at optimising the dose range and refining the threshold at which DAOY cell lines are sensitive to DT treatment. By elucidating the mechanisms underlying this delayed response to DT, it is possible to uncover the intrinsic features of DAOY cells that confer the observed reduced sensitivity. Identifying these mechanisms will be crucial to enhancing the interpretability of the DR/DT system in DAOY cells. It could facilitate the development of models explaining the reasons behind increased resistance, which may potentially be applied to other SHH cell lines. Ultimately, understanding the mechanisms behind DAOY observed resistance is vital for improving the efficacy of the DTR/DT model across SHH medulloblastoma cell lines.

4.6.4 Mechanistic Explanations for Reduced Efficacy

Several mechanistic explanations may account for the reduced sensitivity observed in DAOY-derived lines. Prior studies have noted the apparent fragility of DAOY cell lines in terms of gene expression (van Essen et al., 2024), proliferation ability (Smirnova et al., 2016), and the size and fragility of spheroids, as well as their higher susceptibility to destruction during manipulations after multiple passages (Zanini et al., 2013). The effective functioning of the DTR/DT system depends on the stable expression of DTR at the membrane (Chenal et al., 2002). Therefore, if DTR expression is unstable, the DT cannot effectively bind, initiate internalisation, and undergo the cytotoxic cascade (Naglich et al., 1992).

Furthermore, fluctuations in membrane lipid composition across tumour subtypes may affect the ability of DTR to embed into the membrane, ultimately impairing DTR availability for DT binding (Swinnen et al., 2019). Furthermore, the stress of repeated passaging and the manipulation of 3D cultures may further affect the viability of the cell lines, potentially causing heterogeneity within the population and resulting in inconsistent DTR expression across 3D conditions (Hirschhaeuser et al., 2010). While it is generally acknowledged that DT is functionally potent even at low concentrations (Collier, 2001), any variations in receptor density could disproportionately influence its cytotoxic efficacy.

Moreover, even if the DTR is adequately expressed, intrinsic differences specific to DAOY cells, such as membrane composition or endocytosis pathways, may influence DT uptake and its cytotoxic effects, compared to ONS-76. DT exploits endocytosis processes to enter the cell through a translocation domain that enables it to cross the cell membrane (Hayat, 2016). After binding to receptors, the toxin is endocytosed and

transferred to the Golgi apparatus, where it exerts its toxic effect by inhibiting protein synthesis (Hayat, 2016) or by inhibiting translation arrest, which leads to cell death (Collier, 2001). DAOY cells may differ from ONS-76 in terms of their intracellular mechanistic steps, specifically in altered endocytic vesicle acidification or inefficient vesicle trafficking, which would thereby reduce the composition of DT released into the cytosol (Sandvig & van Deurs, 2002).

Furthermore, an additional potential explanation for the diminished efficacy of the DTR/DT model, concerning ONS-76-derived cell lines, pertains to the apoptotic capabilities of medulloblastoma lines, which are frequently characterised by dysregulated apoptotic pathways (Castellino et al., 2007). Medulloblastoma cell lines show upregulation of anti-apoptotic proteins such as BCL-XL and MCL-1, which serve distinct protective functions (Fitzgerald et al., 2023), in response to pro-apoptotic signals. The increased expression of these proteins confers different yet complementary protective roles, as demonstrated by Fitzgerald et al. (2023). Specifically, MCL-1 inhibits early caspase activation, whereas BCL-XL obstructs the activation of BAX and BAK, thereby enhancing resistance to apoptotic signals triggered by external factors, such as cytotoxic agents. In the context of the DTR/DT model, even if DT is internalised, the downstream apoptotic cascade may be attenuated, potentially elucidating the low levels of cytotoxicity observed in DAOY cells (Fulda, 2009).

Taken together, these considerations suggest that the diminished responsiveness of DAOY cells to the DTR/DT model is attributable not to a solitary interaction but rather to a synergistic effect of multiple factors that are inherent to DAOY cell lines. Understanding the roles of both intrinsic and extrinsic biological factors will be essential to optimise the DTR/DT system for use in DAOY cells and to broaden its translational potential.

4.6.5 Impact of 3D Architecture on Cytotoxic Response

The utilisation of three-dimensional (3D) architecture within the DTR/DT model did not uniformly enhance sensitivity to DT. Whilst ONS-76 spheroids exhibited a robust response even at low doses, DAOY spheroids displayed a much more gradual decline in cell viability, with statistical significance only manifesting at considerably higher concentrations.

It is well established that not all cell lines react equally to external stimuli in 3D environments; some exhibit more in vivo-like behaviours that emulate the tumour microenvironment, whereas others retain basic resistance (Duval et al., 2017; Edmondson et al., 2014). In this study, DAOY cell lines align with the latter group, where apoptotic resistance is present in low doses across various architectural conditions, thereby rendering the DTR model less effective at these concentrations.

In summary, DAOY cells exhibited a significant and robust cytotoxic response following DT treatment; however, this effect was only observed at concentrations of 12 ng/ml or

higher. Conversely, ONS-76 spheroids exhibited potent cytotoxic responses at early doses. These findings underscore the differential sensitivity of the DTR-DT model across cell lines. Although it was efficacious in inducing cytotoxicity in both cell lines, its effectiveness was highly dose- and cell line-dependent. Such variability in responses highlights the importance of validating this system across SHH medulloblastoma subtypes to develop a more comprehensive model that enhances therapeutic relevance.

4.7 Functional Efficacy of the DTR/DT System in 2D and 3D Models

The primary aim of this study was to evaluate the functional efficacy and specificity of the DTR/DT system in inducing targeted cell death in transfected cell populations, while minimising off-target effects. This model relies on the selective specificity of DTR-GFP-expressing cells by DT, a mechanism that has been widely employed in vivo for the selective ablation of cells in developmental and disease contexts (Cerpa et al., 2014; Okuyama et al., 2010). However, upon reviewing the literature, few studies have assessed the use of DTR/DT models in medulloblastoma models. This scarcity of data underscores the potential value of investigating this novel approach.

4.7.1 Comparison with Alternative Ablation Systems

Compared to other widely adopted systems for selective ablation, such as the herpes simplex virus thymidine kinase system (HSV-TK), the DTR/DT approach demonstrates superior experimental efficacy. DT-mediated ablation occurs rapidly (Mafalda et al., 2017), with significant levels of irreversible cell death observed within 48 hours of exposure, as shown in this experiment. This aligns with DT's well-studied rapid mechanism of action, which swiftly halts protein synthesis, leading to cell death through the inactivation of a critical translation factor in a single step. Additionally, this highlights the efficiency of DT as a system for cell ablation (Naglich, Metherall, et al., 1992).

Conversely, systems such as HSV-TK/GCV result in a significantly slower cell ablation process due to the slow uptake and multiple enzymatic steps required to convert GCV into its toxic form (Haberkorn et al., 1998). This multi-step process in the HSV-TK/GCV system (Rubsam et al., 1999), as opposed to the one-step apoptotic effect of the DTR system (Liu et al., 2019), directly limits the system's efficiency. Furthermore, the HSV-TK system exhibits a 'Bystander Effect,' which introduces inherent limitations relative to the DTR/DT system, as HSV-TK can induce cytotoxicity in non-transduced cells through transduced cells (Wang et al., 2004), thereby constraining its capacity to effectively ablate an entire target cell population.

Critically, while the DTR/DT model offers advantages for demonstrating population-specific ablation, its implementation is constrained by the requirement for uniform DTR

expression, which may allow subpopulations with low expression to escape ablation altogether. Furthermore, the model's significant reliance on genetic modification limits its immediate applicability to murine systems. Compared with the HSV-TK system, which exhibits some ablation of heterogeneous populations, albeit with a slower time course and potential bystander effects. Therefore, the DTR/DT system is most advantageous when absolute cell-specific ablation is needed with minimal off-target effects.

4.7.2 Implications for Precise Ablation

The specificity and efficacy of the DTR/DT model system, as demonstrated in this experiment, possess considerable implications for the progression of therapeutic strategies capable of precisely targeting specific populations while minimising off-target effects within a tumour environment. The swift cytotoxic effects and strict selectivity of the system render it highly valuable and dependable for functional studies that involve the precise elimination of cell populations. Its exceptional specificity and rapid action, coupled with a complete lack of bystander effects, clearly establish this as an advantageous system for the development of precision ablation therapies.

4.7.3 Cell Line Dependent Efficacy of the DTR/DT model

These results further emphasise the necessity of varying functionalities of genetic ablation systems across various cellular lines. Although both ONS-76 and DAOY are classified as Shh subtypes, they demonstrate markedly different cytotoxic responses, thereby illustrating that while the DTR/DT approach is generally considered robust and specific, its efficacy is not universally assured (Ruedl & Jung, 2018).

It is imperative to account for differences in transfection dynamics, receptor biology, and apoptotic susceptibility among individual cell lines to ensure proper functional validation. The unanticipated resistance observed at low doses in DAOY cells serves as a cautionary reminder against overgeneralising successful ablation strategies, which often presume efficacy across similar cell types. Consequently, the functional validation of the DTR/DT model should be conducted on a case-by-case basis, supported by comprehensive assessments of each cell line to verify system integrity and response dynamics.

4.7.4 Recommendations for Future Studies: Functional Validity

Future studies could include additional quantitative assessments such as Western Blot analyses to quantify DTR receptor density (Saumell-Esnaola et al., 2022). This would aid in establishing a connection between receptor density and functional responsiveness, specifically regarding cytotoxicity to DT. By correlating receptor density with cell viability, researchers can better determine the functional thresholds necessary for

effective ablation and investigate potential mechanisms of resistance in specific cell lines (Saumell-Esnaola et al., 2022). Incorporating these validation steps into experimental design enhances the specificity of the model system and increases the reliability of cell-specific ablation models.

4.8 Limitations and Future Directions

While the current study serves as a valuable instrument to facilitate the development of effective utilisation of the DTR/DT system in examining the long-term effects of cancer treatment, several limitations restrict the generalisability and depth of our conclusions.

4.8.1 In Vitro Model Limitations

Firstly, the use of in vitro models to develop an effective ablation system inherently constrains the translational applicability of our findings. While it is essential to acknowledge that 3D spheroid cultures can better replicate the complex tumour microenvironment compared to traditional 2D cultures, it is equally important to note the limitations associated with in vitro cell lines and subsequent models (Ghallab, 2013).

These models cannot fully encompass the intricacies of the tumour microenvironment as it exists in patients, particularly concerning the interactions of immune cells (Mu et al., 2023), the presence and organisation of vascular networks (Murphy & Allenby, 2023), and the extracellular matrix (ECM) (Tang & Liu, 2023). These components play vital and complex roles in tumour proliferation, influencing how tumours respond to various treatments, including drug therapies, and significantly impacting long-term outcomes following different treatment modalities (Wang et al., 2024).

Furthermore, our model is limited by its failure to replicate the tumour microenvironment, particularly the immune and neural components, which are essential for establishing tumour-host interactions in response to chemotherapeutic insult. The absence of immune cells restricts the significance of our conclusions regarding systemic and neural immune effects.

Consequently, this limitation means that the extent to which our in vivo observations can be generalised remains limited. Further studies utilising more complex and representative models, such as an in vivo system incorporating tumour organoids (Idrisova et al., 2022), are necessary to validate and extend these findings to ensure downstream applicability in clinical settings, ultimately aiding in the development of more effective and targeted therapeutic interventions and models to study the long-term effects of treatment.

Furthermore, the ONS-76 and DAOY cell lines, although commonly utilised and extensively characterised within the scientific community, are inherently limited in their capacity to be generalised to other subtypes of medulloblastoma. Although both cell lines are derived from the Shh subgroup, this restricts the scope of our observations and implies that findings derived from them may not be fully applicable to the full

spectrum of medulloblastoma subtypes. Our research demonstrates that cell line-specific differences are inherently present among different cell lines, highlighting the importance of considering such variability when extrapolating results.

4.8.2 Stress of Repeated Passaging

Another significant limitation of this study is the cellular stress caused by repeated passaging, which applies intense selective pressures that may alter cellular characteristics over time, potentially leading to cellular senescence (Herr et al., 2024). Each passage subjects the cell lines to DNA damage, resulting in different levels of genomic instability (Handl et al., 2020). Moreover, during repeated passaging, artificial selection might lead to the expansion of more resilient clones. At the same time, more sensitive cells are lost due to competition, resulting in overrepresented subpopulations that are less representative of the actual tumour characteristics (Podsednik et al., 2024). Therefore, such observed differences in drug sensitivity between cell lines may reflect the stress caused by repeated passaging, rather than the intrinsic differences in cell line sensitivity. Accounting for the effects of long-term repeated passaging is therefore crucial when conducting longitudinal studies to ensure reproducibility.

4.8.3 Restricted Scope of Study

Although the developed DTR/DT model provides a solid foundation for modelling survivorship scenarios and long-term treatment effects, it does not currently account for longer-term downstream outcomes such as persistent cognitive deficits or chronic pain. These outcomes could be explored with a more complex model that includes interactions with neural and non-neural cell populations. Similarly, this study does not cover potential off-target signalling effects, like subtle disturbances in the EGFR pathway or long-term adaptive responses that may become significant over time. Therefore, the limited scope of the current study reflects a deliberate focus on developing a reliable, robust platform for selective tumour cell ablation and long-term modelling. Future research should aim to extend the model to replicate long-term neurological outcomes.

4.8.4 Limited Duration of Monitoring and Late Effects

Whilst our DTR/DT system allows insights into the immediate effects of cancer treatment through selective cell ablation, its applications are limited by the duration of monitoring we carried out. This timeframe, therefore, may be viewed as insufficient to capture the long-term effects of treatment that would otherwise manifest over extended periods.

For example, therapeutic resistance is a complex and multifaceted phenomenon in which cancer cells, over time, develop to adapt to the selective pressures imposed by therapy. This adaptive process allows tumour cells to survive and proliferate despite ongoing treatment, thereby contributing to disease progression and treatment failure

(Lei et al., 2023). This phenomenon can be attributed to a variety of factors, which have been extensively characterised and include genetic alterations, epigenetic modifications, and changes within the tumour microenvironment (Lei et al., 2023). Within the context of our study, due to the short period, it is possible that resistant clones may not have had sufficient time to emerge. This limitation could lead to an underestimation of the actual long-term efficacy of the DTR/DT system, as resistant strains develop or become more apparent over a longer duration. By addressing this limitation, further research could bridge the gap between the short-term effects observed in in vitro studies and the long-term outcomes seen in clinical settings, thereby enhancing the understanding of the detrimental effects of therapeutic interventions. The effects of cancer treatment, including surgery and chemotherapy, can manifest months to years after treatment concludes (Lustberg et al., 2023), impacting neurological and cognitive functions (Ahles & Root, 2018). These include cognitive decline (Koppelmans et al., 2012), slower processing speed, and reduced psychomotor function, which also impact brain metabolism and functional connectivity (Dias-Carvalho et al., 2021).

In paediatric populations, the effects of chemotherapy are particularly long-lasting, leading to significant reductions in QoL mainly due to the neurotoxic effects of chemotherapeutic agents (Marusak et al., 2017). The so-called “double hit,” as described by Marusak et al. (2017), combines the harmful effects of early life exposure to pain and treatment methods, both of which aim to exacerbate the adverse side effects that alter neural development as well as cognitive, behavioural, and emotional outcomes. Emerging research attributes this to the injurious effects of chemotherapeutic interventions that directly harm the developing brain (Butler & Haser, 2006; Cheung & Krull, 2015), leading to alterations in cognitive and affective processing (Thomason & Marusak, 2017). The short-term monitoring of our experiment, therefore, does not detect such long-term detrimental effects.

4.8.5 The Cerebellum: A Critical Vulnerable Site of Damage

The cerebellum is a common site of medulloblastoma origin (Brandes et al., 2009), making it a susceptible target for damage stemming from chemotherapeutic approaches, which can lead to a variety of cognitive and neurodevelopmental deficits (Nguyen & Ehrlich, 2020). Given the highly proliferative nature of granule neuron progenitor cells within the cerebellum, they are highly vulnerable to damage and disruption, especially during the early stages of life (Wood & RJ Youle, 1995).

Therefore, treatment that acts within the paediatric brain leaves individuals especially vulnerable (Marusak et al., 2017), meaning the timing of treatment is critical. Chemotherapeutic approaches can negatively impact the levels of granule progenitor cells, leading to disrupted synaptic networks (Dietrich et al.2006). This irreversible loss of valuable neurons during a critical developmental stage sets the stage for long-lasting effects on neurodevelopment. Furthermore, the loss of granule cells further disrupts

cerebellar circuits by impairing Purkinje cell development, thereby reducing the inhibitory output that typically results from the interactions between Purkinje and granule cells, which causes impaired motor and cognitive processing (van der Heijden & Sillitoe, 2021). Many of these impairments are late-emerging, becoming more pronounced as an individual develops through schooling and social interactions (Schagen & Wefel, 2013).

As described by Marusak et al. (2017), the double hit theory precisely encapsulates the concept of late emerging effects of treatment: combining the implications of early life pain with cancer treatment effects, leading to an exaggerated plethora of long-term deficits. Further models, which take into account the long-term effects, must be developed to help characterise the mechanism behind this, perhaps even elucidating a genetic link between those who have more far-reaching late-stage consequences (Kateryna Petrykey et al., 2019).

Gaining a deeper understanding of the cerebellum's highly vulnerable nature is essential to reducing deficits during the survivorship phase. By uncovering the mechanisms underlying these late-emerging deficits, early intervention strategies can be tailored to the unique profile of cerebellar damage, supporting the development of less neurotoxic therapies that not only improve survival but also enhance the QoL for survivors.

4.8.6 Translational Gaps- Bridging Experimental and Clinical Contexts

Despite the promising findings of this study, the present DTR/DT system is not directly translatable to human populations. It is essential to acknowledge that the proposed model has limitations in its clinical implications. It is becoming increasingly clear from emerging models that the developing brain is uniquely vulnerable to cytotoxic treatments (Ikonomidou, 2018), which is why the present study and its results are of significant importance. The results of this study lay the foundation for generating a model that can mimic the precise timing of intervention and the progression of cognitive decline, thereby uncovering the mechanistic links underlying the effects of early-life chemo exposure and late-emerging neurodevelopmental outcomes.

It is important to note that these findings do not provide an immediate approach for the development of targeted cancer cell ablation. However, they enable the bridging of the mechanistic conclusions with clinical observations. Our developed DTR/DT system enables the examination of how chemotherapeutic damage translates into long-term effects, providing a connection between short-term insults and neurodevelopmental outcomes. Importantly, this research demonstrates the potential for clinical application to benefit patients who suffer disproportionate long-term deficits following chemotherapy, offering a pathway to more targeted and effective interventions.

4.8.7 Integration with Longitudinal Models

This system has high potential for longitudinal monitoring. By extending the experimental timeline, it is possible to monitor the manifestation of delayed deficits and perhaps elucidate the mechanisms underlying them. This, therefore, could be utilised to develop early life stage therapeutic interventions that can mitigate damage caused by chemotherapeutic approaches. Taken together, these findings suggest that the use of the DTR/DT system for specific cell ablation could provide unprecedented insights into delayed neurodevelopmental trajectories.

Despite these limitations, the DTR/DT platform developed in this study lays the groundwork for future investigations into the long-term neurological deficits caused by cancer treatment, an area that needs urgent attention. For example, the development of an organoid system derived from human induced pluripotent stem cells could generate structures that mimic brain areas containing both tumour and non-tumour populations (Tong et al., 2024). Within this context, our system could be utilised to exhibit precise tumour ablation, followed by long-term observation to assess changes in electrophysiological activity and neuronal connectivity (Ma et al., 2021). Such utilisation of this more physiologically relevant approach (Ma et al., 2021) could help study the effects of tumour ablation or chemotherapeutic agents and their impact on the integrity of neural networks that cause cognitive decline.

Importantly, this approach would enable researchers to explore in greater detail the effects of exposure to cancer treatment-induced neurotoxicity within a tumour-like environment. Furthermore, with further development, this approach could be designed to integrate seamlessly with advanced molecular profiling techniques. These techniques would enable the precise identification of cell-type-specific epigenetic and translational changes, shedding light on the mechanisms that lead to long-term dysfunction. Such insights could significantly advance our understanding of treatment-related neurotoxicity and inform the development of targeted interventions.

Moreover, the long-term approach could help address specific brain areas that are particularly vulnerable to cancer therapies and play a significant role in its cognitive decline. In particular, the cerebellum, which is an area of origin for some medulloblastomas (Aljoghaiman et al., 2018), contains highly proliferative granule neuron progenitor cells, making them highly sensitive to chemotherapeutic agents (Dietrich et al., 2006; Fraser et al., 2019).

5.0 Conclusion

The findings from this research offer compelling evidence to support the use of DT-mediated ablation within the framework of SHH-derived medulloblastoma models, owing to its particular attributes, albeit limited to three-dimensional culture settings. This represents a critical finding, underscoring the importance of our investigation, which, in contrast to prior studies (Lim et al., 2022; Saito et al., 2001) that have only established the DTR/DT model in 2D in vitro environments, is the pioneering effort to utilise 3D culture models for a more physiologically pertinent approach to medulloblastoma. Such an advancement is crucial for the development of novel therapeutic strategies that aim to preserve healthy, non-cancerous surrounding cells.

The data here support the usefulness of the ONS-DTR-GFP and DAOY-DTR-GFP spheroid model as effective platforms for population-specific ablation using DT. It is essential to note that ONS-76 DTR-GFP-Expressing spheroid models exhibited an obvious cytotoxic response to DT at low doses, confirming the utility of the DTR/DT system within the framework of ONS-76 cell lines for selective ablation. In stark contrast, DAOY cells exhibited relative resistance to DT cytotoxicity, with significant cytotoxic effects only observed at doses higher than 12 ng/mL. This model provides a vital foundation for a system that is more physiologically relevant, thereby facilitating the development of targeted therapeutic interventions and the study of the long-term effects of cancer treatments.

Furthermore, this study illustrates the successful development of an optimised process for the selection, transduction, and three-dimensional modelling of ONS-76 and DAOY medulloblastoma cell lines. The DTR/DT system itself facilitates the precise ablation of specific cell populations in DTR-GFP-expressing ONS-76 and DAOY spheroids, with responses contingent upon culture dimensionality, dose of DT and cell line characteristics. Conversely, DAOY cell lines, in stark contrast, exhibit slight resistance to DT at lower concentrations irrespective of culture dimensionality, thereby emphasising the importance of validation for specific cell lines. Consequently, this work establishes a groundbreaking foundation for future investigations to utilise DTR/DT-mediated ablation in targeting specific populations of medulloblastomas.

The findings of this study provide a promising foundation for developing a system to investigate the long-term neurocognitive effects of survivorship following treatment for medulloblastoma. These results carry implications of both scientific and societal significance. Mechanistically, although this study is not directly translatable to humans, it enables controlled ablation of specific cell populations, facilitating the investigation of long-term effects that would otherwise be difficult to isolate in vivo.

From a scientific standpoint, this DTR/DT model can inform the development of future neuroprotective strategies for medulloblastoma treatment that mitigate the neurocognitive deficits caused by neuronal vulnerability during therapy. These insights can be utilised for the development of interventions aimed at reducing cognitive decline, particularly within paediatric populations where developmental considerations of treatment are crucial. Moreover, the development of cell-line-specific techniques,

while considering dimensional effects, emphasises the need for tailoring experimental approaches to specific biological contexts, providing a robust framework for a reproducible and physiologically relevant model.

From a societal and ethical standpoint, these findings emphasise the importance of responsible interpretation of research whilst avoiding overstatement of its translational applicability. This research, whilst valuable, does not replicate the complex interactions present in clinical settings, including immunological responses, metabolic processes, and the impact of socio-environmental factors. Furthermore, this study highlights the need for responsible research, ensuring outcomes are contextualised within a broader societal framework.

Finally, this model provides a platform to explore targeted ablation, which is ethically justifiable as it informs neuroprotective techniques that do not cause unnecessary suffering, even within animal models. By facilitating the development of this DTR/DT system, this contributes to the refinement and reduction principles of the three scientific R's proposed by Russell and Burch (1959), offering a platform to decrease reliance on harmful cancer treatment techniques whilst generating meaningful biological insights. Collectively, the broader implications of this work are far-reaching, providing a solid foundation for subsequent translational and applied research.

5.1 Ethical Considerations

The focus of this study was to assess the vulnerability that stems from treatment. Whilst the DTR/DT model is a valuable tool, it is equally important to address the ethical implications of employing such a reductionist system in research. A primary cause of ethical concern within the DTR/DT system is the development of a system that causes cognitive impairment through a reductionist approach. The current system oversimplifies the complex interactions that stem from chemotherapeutic effects, such as immune cell responses, damage to white matter integrity, and loss of synaptic functionality. This, therefore, risks oversimplifying the survivor's experience, which is actually shaped by a complex interplay of various emotional, social, and economic factors that can differ significantly from one individual to another (Sheikh-Wu et al., 2023).

There is a growing need to recognise this complexity, leaving researchers with an ethical obligation to interpret findings and design future studies with great care, thoroughly acknowledging these limitations to prevent deterministic or overly generalised predictions of cognitive outcomes that would otherwise risk harming the patient. Alternatively, future research must adopt a more comprehensive approach to survivorship care, one that considers the diverse and multifaceted factors influencing an individual's unique survivorship journey, including psychological effects, social support networks, economic stability, and access to healthcare resources.

5.2 Concluding Remarks

This study aimed to develop a model that utilises DT for selective cell ablation in medulloblastomas, focusing on its potential for long-term studies of treatment effects. The development of this refined DTR/DT model signifies a significant methodological advancement for longitudinal studies of survivorship. It was found that DT induced specific, substantial cytotoxicity across both ONS-76 and DAOY cell lines; however, this was dose-dependent and varied between cell lines, with ONS-76 spheroids exhibiting sensitivity to lower doses and DAOY cell lines exhibiting relative resistance. Overall, significantly elevated levels of cell death were observed, specifically in both DTR-GFP-expressing subpopulations, although there was variability in growth and inherent resistance between cell lines, meaning that ablation may not be entirely uniform. These results also highlight inherent population-specific differences within cell lines of the same subgroup, emphasising the need to elucidate these variations to develop a more targeted system.

It is evident that using 3D spheroid models further enhances the study's validity and translational relevance by providing a physiologically relevant environment that mirrors cell-cell interactions, nutrient gradients, and spatial organisation. It was confirmed that DTR expression did not affect basal growth characteristics in 3D cultures, ensuring that observed effects could not be attributed to transfection but instead relied solely on DT-induced, selective cytotoxicity.

Overall, this study demonstrates the potential of the DTR/DT system as a robust, precise, and adaptable method for targeted cell ablation in medulloblastoma models. The demonstrated effectiveness in ONS-76 and DAOY highlight the system’s potential for future mechanistic investigations into the long-term effects of treatment-related cognitive deficits. Ultimately, the findings presented here represent a crucial step toward developing experimental systems that can inform the identification of neuroprotective strategies and enhance survivorship care.

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