**Advancing Multi-Target Neuronal Differentiation (MTND) Therapy in the treatment of IDH-wildtype and IDH-mutant gliomas**

CANC 440

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**Abbreviation List:**

GBM: Glioblastoma Multiforme

IDH: isocitrate dehydrogenase

IDH-wt: IDH-wildtype

IDH-mutant: IDH-mutant

TMZ: temozolomide

CSC: cancer stem cell

MTND: Multi-target neuronal differentiation treatment

OS: Overall Survival

1. **Scientific Abstract**

Glioblastoma multiforme (GBM) is the most common primary brain tumour. Median overall survival is typically 5-23 months, representing the lowest long-term survival rate of all cancers.1 First-line treatment involves surgical resection, followed by temozolomide (TMZ) chemotherapy and radiation; unfortunately, GBMs are heterogenous and frequently recur.6 Knowledge of GBM carcinogenesis has shifted to fit a cancer stem-cell (CSC) model, in which select tumour cells are highly tumorigenic due to their self-renewal, proliferation, and multipotentiality.10 Following the CSC model, differentiation therapy has emerged as a method to reduce tumour proliferation, with fewer side effects from cytotoxicity. Rather than killing CSCs, differentiation therapy coaxes neural precursors into terminal differentiation, so that they lose their proliferative ability.12 Differentiation therapy has been remarkably successful in treating acute promyelocytic leukemia, in which retinoic acid restores myeloid cell differentiation. Consequently, researchers have explored the potential use of differentiation therapy in brain tumours. Recently, Hu et al. (2023) tested eleven small molecules known to induce differentiation and established a multi-target neural differentiation (MTND) therapeutic cocktail, composed of Forskolin, Dorsomorphin, Purmorphamine, CHIR 99021, and P7C3-A20.16 MTND induces rapid reprogramming of IDH-wt glioma cells into neurons.16 Experiments from mouse xenograft models of IDH-wt glioma compared MTND vs. TMZ treatment and demonstrated that the MTND-treated group developed much smaller tumours than the TMZ-treated group.16

The proposed study will further elucidate MTND’s potential as differentiation therapy in IDH-mutant tumours, as well as its impact on OS. Cell cultures representing IDH-wt and IDH-mutant gliomas will be generated for in vitro studies. Two types of orthotopic xenograft models of GBM will be generated to represent IDH-wild-type (n=15) vs IDH-mutant gliomas (n=15). The experimental groups will receive TMZ or MTND, while the control groups will receive saline, via stereotactic injection (n=5 per group). We hypothesize that MTND therapy will similarly induce differentiation in IDH-wt and IDH-mutant tumour cells in in vitro models. Furthermore, MTND therapy will prolong OS in mouse models of glioma, compared to TMZ or control. Finally, MTND therapy will induce differentiation within in vivo models.

1. **GBM: Current Landscape**

Glioblastoma multiforme (GBM) is the most common primary central nervous system (CNS) tumour, as well as the most malignant. Median overall survival (OS) is typically 5-23 months, representing the lowest long-term survival rate of all cancers.1 Upon suspicion of GBM due to symptoms such as headache or cognitive impairment, patients undergo an MRI (magnetic resonance imaging) scan to locate the tumour, then a biopsy for pathological analysis. The first grade of GBM – Grade 1 – is surgically curable; however, Grade II-IV tumours are highly infiltrative and invariably progress to later stages.2 GBM tumours can be classified as IDH wildtype or IDH-mutant: IDH refers to isocitrate dehydrogenase, which catalyzes the decarboxylation of isocitrate in the citric acid cycle. In gliomas, IDH mutations typically involve an arginine-to-histidine conversion at position 132 (R132H), affecting the catalytic site of IDH1.3 IDH-mutant glioma generally exhibits a more favourable prognosis;4 although the molecular mechanisms underlying this advantage is unclear, it is suggested that IDH-mutation reduces tumour cells’ ability to scavenge oxygen species, causing tumours to be less proliferative and more susceptible to chemotherapy.5

First-line treatment involves surgical resection, followed by temozolomide (TMZ) chemotherapy and radiation; unfortunately, GBMs are heterogenous and frequently recur. TMZ is an alkylating agent that is orally administered and capable of crossing the blood-brain barrier.6 It attaches methyl groups to guanine and adenine nucleotide bases, generating the cytotoxic bases O6-methyguanine, N3-methyladenine, and N7-methylguanine, which trigger cell death as the cell attempts to replicate DNA. However, malignant tumours can develop resistance to TMZ by activating DNA repair pathways, such as O6-methylguanine-DNA-methyltransferase (MGMT), mismatched repair, and base excision repair.6 TMZ comes with significant side effects, such as nausea, anorexia, and thrombocytopenia.7 TMZ has been found to be more effective in patients with MGMT methylation (i.e., silencing), but only 35-45% of patients with Grade III-IV glioma exhibit this feature.8

A single tumour is comprised of multiple types of cells, a phenomenon termed intra-tumour heterogeneity: because not all cells respond to one treatment, tumour heterogeneity forms the foundation of treatment resistance.9 GBMs are particularly heterogenous, as tumours are often comprised of clonal and subclonal differentiated cells, stem cells, and non-tumour endothelial/inflammatory cells.9 Knowledge of GBM carcinogenesis has shifted to fit a cancer stem-cell (CSC) model, in which select tumour cells are highly tumorigenic due to their self-renewal, proliferation, and multipotential characteristics.10 CSCs generate tumour heterogeneity by establishing a range of cell types, which can differentiate and de-differentiate; but as cells gain greater stemness, they gain greater potential to proliferate and to generate cells that can evade treatment.11 If it is possible to ‘force’ CSCs to differentiate into a terminal, postmitotic state, they will lose their proliferative ability, helping to alleviate dysregulated tumour growth and resistance.10

From this line of thought, differentiation therapy has emerged as a method to reduce tumour proliferation, with fewer side effects caused from cytotoxic effects of chemotherapy. Rather than killing CSCs, differentiation therapy coaxes neural precursors into terminal differentiation, so that they lose their proliferative ability.12 Differentiation therapy has been applied with striking success in the treatment of acute promyelocytic leukemia, in which retinoic acid restores myeloid cell differentiation;12 this therapy has led to complete remission and OS rates greater than 95%.13 In brain tumours, differentiation therapy has shown burgeoning promise: cis-retinoic acid may induce differentiation of neuroblastoma cells,14 while bone morphogenic proteins can induce differentiation of neural precursors into astrocytes.15

Recently, Hu et al. (2023) tested eleven small molecules known to induce differentiation and established a multi-target neural differentiation (MTND) therapeutic cocktail, composed of 10 µM Forskolin, 1 µM Dorsomorphin, 1 µM Purmorphamine, 3 µM CHIR 99021, and 3 µM P7C3-A20.16 These components provide a multi-target approach to address different signalling pathways involved in endogenous differentiation of glioma to terminal neurons. Forskolin activates adenylate cyclase to increase levels of cyclic AMP, which then promotes neural maturation pathways; it also activates protein kinase to induce cell cycle arrest in proliferating cells.17 Dorsomorphin inhibits SMAD transcription factors, thereby promoting differentiation of embryonic stem cells into neural lineages.18 Purmorphamine activates the Sonic hedgehog (Shh) pathway involved in neurogenesis and neural patterning.19 CHIR 99021 is a glycogen synthase kinase 3β (GSK-3β) inhibitor, which facilitates the Wnt-β-catenin pathway to promote differentiation.20 Finally, P7C3-A20 is a neuroprotective compound that inhibits the death of mature neurons.21 The authors established in vitro models from the specimens of three clinical patients with IDH-wt glioma, and they found that MTND induces rapid reprogramming of glioma cells into neurons.16 They also conducted experiments from mouse models of glioma to MTND vs. TMZ treatment over 10 days and demonstrated that the MTND-treated group developed much smaller tumours than the control or TMZ-treated groups.16 This result promises significant potential in treating GBM with differentiation therapy to treat GBM; however, there are open questions on whether MTND treatment is effective in IDH-mutant gliomas, and whether MTND treatment can actively improve survival outcomes.

1. **Hypothesis and Aims**

This project intends to advance preclinical research on MTND therapy by exploring its efficacy in IDH-mutant tumours and its impact on prolonging survival. Given that IDH mutations offer a favourable prognosis due to increasing receptivity to TMZ therapy,5 it does not seem likely that IDH mutation status would interfere with the mechanism of MTND; therefore, it is hypothesized that MTND promotes differentiation similarly on IDH-wt and IDH-mutant glioma cells. Furthermore, MTND treatment will likely prolong the OS of mice with IDH-wt or IDH-mutant glioma, compared to TMZ or control treatment. Finally, MTND treatment will likely promote loss of stem cell markers and gain of differentiation markers, which indicates that it acts similarly within in in vivo and in vitro models. Our aims are as follows:

* **Aim 1:** Establish in vitro models of IDH-wt and IDH1-mutant gliomas and treat with MTND, to replicate findings from Hu et al. (2023) in IDH-wt cells and to determine if MTND can promote similar differentiation effects in IDH-mutant cells.
* **Aim 2:** Administer control (saline), TMZ treatment, or MTND treatment to patient-derived xenograft mouse models of IDH-wt and IDH-mutant GBM, to determine if MTND treatment improves OS above control and/or TMZ.
* **Aim 3:** Extract and section tumours after each experiment’s end to conduct immunohistochemistry for stem cell and differentiation markers, to determine if MTND is acting to convert stem cells into differentiated neurons within in vivo models.

1. **Aim 1 Methodology**

Cell lines representing IDH-wt and IDH-mutant gliomas will be purchased from the ATCC repository.22, 23 To visualize if MTND therapy induces similar differentiation programs in IDH-wt and IDH-mutant cells, cells will be cultured in medium vs. medium with MTND, then imaged with bright field microscopy.

To measure cell proliferation, an EdU assay will be performed (Click-iT EdU Cell Proliferation Kit for Imaging; Invitrogen).16 IDH-wt and IDH-mutant cells will be seeded onto coverslips and treated with control (0.1% DMSO), 200 µL TMZ, or 200 µL MTND for 48 hours, then incubated with 10 µM EdU (a substance that is incorporated into newly synthesized DNA) for 24 hours.16 Finally, cells will be fixated and stained with Hoechst, then imaged with fluorescent microscopy, to determine proliferation rate for all conditions.16

Success will be determined by visualizing the morphology of IDH-wt and IDH-mutant cells: if they resemble matured neurons, then MTND treatment is effective in inducing differentiation within both tumour subtypes. Furthermore, success will be determined by using statistical t-tests to compare proliferation rates between control, TMZ, and MTND conditions for IDH-wt and IDH-mutant cells in the EdU assay: MTND treatment is expected to significantly reduce proliferation compared to TMZ and control. This result would serve to replicate findings from Hu et al. (2023) in IDH-wt tumours, and to extend those findings to IDH-mutant tumours.

1. **Aim 2 Methodology**

Two mouse models will be generated from female BALB/c immunodeficient mice to represent IDH-wt and IDH-mutant tumours, following xenograft models used by Esmaeili et al. (2014). The E434 xenograft will be used to generate IDH-wt models, while the E438 xenograft will be used to generate IDH-mutant (IDH1-R132H) models; both xenografts originate from biopsies of patients with high-grade glioma.3, 24 Glioma will be transplanted orthotopically by injecting tumour cells (5x105 cells in 5 µL) into the lateral ventricles.16 A stereotactic tube will be implanted and fixed to the mouse’s skull, which will allow the mouse to move freely, while control (saline), TMZ, or MTND treatment will be administered once per day.16 Treatment will continue for the duration of the mouse’s survival, up to a maximum of 120 days (when mice will be anesthetized and terminated). Overall, 30 models will be generated for this study (15 IDH-wt mice and 15 IDH-mutant mice; within each group, 5 mice allocated to control, TMZ, or MTND).

Success will be determined by plotting a Kaplan-Meier survival curve, indicating time-to-death for control, TMZ, and MTND-treated mice. In both IDH-wt and IDH-mutant models, we expect MTND mice to survive for longer than TMZ mice, and for TMZ mice to survive for longer than control mice.

1. **Aim 3 Methodology**

At the end of each model’s experiment (i.e., either by death or at the 120-day conclusion), brain tissues will be removed by craniotomy. The tumour will be isolated and fixed in 4% paraformaldehyde for histological sections. After sectioning, slides will be stained for DAPI, a nuclear stain that enables cell counting; nestin, a cytoskeletal protein frequently expressed in glioma stem cells; for TUJ1, a marker with increased expression during neuronal differentiation.16, 25, 26 Slides will be visualized with fluorescent microscopy.

Success will be determined qualitatively and quantitatively. We expect to observe a visual difference in fluorescent intensity between control, TMZ, and MTND-treated tumours (both IDH-wt and IDH-mutant), in which MTND-treated tumours show greater fluorescence for TUJ1 and lower fluorescence for nestin. Since the tumours will be different sizes, we will also quantify fluorescent intensity (i.e., with ImageJ software)27 normalized by the number of cells (marked by DAPI) on the slide. Again, we expect MTND-treated tumours to demonstrate a greater proportion of TUJ1 fluorescence and a lower proportion of nestin fluorescence.

1. **Significance**

Data generated from this study will build on exciting results of differentiation therapy for GBM. First, this study will demonstrate if MTND treatment is effective for IDH-mutant glioma within in vitro and in vivo models, opening opportunities for further preclinical exploration of differentiation therapy within both subtypes of GBM. As Hu et al. (2023) did not measure long-term survival, this study will demonstrate if reduced tumour burden and neuronal differentiation associated with MTND translates into a long-term survival outcome. Finally, this study will confirm that the mechanism of action of MTND – to induce differentiation in glioma stem cells – is validated not only in cell cultures, but also in mouse models. Future work may build on these results by conducting toxicity and adverse effect studies, before indicating MTND as a novel candidate for investigation in a Phase I clinical trial of GBM. Ultimately, we hope that MTND formulations can be used to treat GBM and improve OS for patients downstream.

1. **Figures**

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| A diagram of a disease treatment  Description automatically generated |

**Figure 1.** Principle ofMTND treatment (figure adapted from Hu et al., 2023).16

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| **a.** Cell Morphology | A diagram of a diagram of a process  Description automatically generated with medium confidence | |
| **b.** EdU proliferation assay | A dropper and a test tube  Description automatically generated | |
| **c.** Expected result for Experiment A (adapted from Hu et al., 2023): differentiation in MTND condition.16  Control:  A close-up of a grey surface  Description automatically generated  MTND:  A close-up of a microscope  Description automatically generated | | **d.** Expected result for Experiment B (adapted from Hu et al., 2023): Lower EdU in MTND.16  EdU assay:  A collage of images of different types of cells  Description automatically generated  T-test comparing proliferation rate:  A graph of different colored bars  Description automatically generated |

**Figure 2.** Schematic of proposed methodology (a, b) and expected results (c, d) for Aim 1.

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| Xenograft models of IDH-wt vs. IDH-mutant glioma; treatment via stereotactic tube   1. **A diagram of a patient's reaction     Description automatically generated** |
| Expected result (figure adapted from Yuan et al., 2018): increased survival for MTND >> TMZ >> control.28   1. A graph of a number     Description automatically generated with medium confidence |

**Figure 3.** Schematic of proposed methodology (a) and expected results (b) for Aim 2. Subjects who do not reach endpoint at the experiment’s end (120 days) are censored.

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| Immunohistochemistry of IDH-wt and IDH-mutant tumours after death in mice treated with control, TMZ, or MTND.   1. A black arrow pointing to a black arrow     Description automatically generated | |
| Expected Result (adapted from Hu et al., 2023): increased TUJ1 and decreased nestin expression in MTND-treated tumours.16   1. **A collage of images of different types of cells     Description automatically generated** | Expected Result (adapted from Hu et al., 2023): increased TUJ1 and decreased nestin expression in MTND-treated tumours.16   1. **A graph of different colored bars     Description automatically generated** |

**Figure 4.** Schematic of proposed methodology (a) and expected results (b, c) for Aim 3.

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Preamble:

This paper was the culminating assignment in CANC 440, a seminar course in my final semester of university. At the start of the course, our professor let us know that we were to propose our cure to cancer, in no less than 12 weeks. Usually, that statement is loaded with irony. For example: lamenting the difficulty of medical school admissions, my pre-med peers would say “I couldn’t get into med school even if I cured cancer.” My non-pre-med peers would not believe that it is possible to cure cancer in this lifetime; it is the achievement of some Einstein not yet born: a 300 IQ, 200th percentile type of person. Really, it’s impossible. I scratched my head a little and made quick calculations in my head, wondering how well I needed to do in the rest of the course for my GPA to survive a probable failure.

Over the course, we had guest lecturers come in to speak about their cancer of interest. They would assign us a paper, which we would read within the week to submit questions. Some of us would then present and lead the seminar on the paper in the next class. As we caught up on the theories, methods, and recent breakthroughs, an idea began to take shape in my head. We discussed cancers in all systems of the body, and we discussed challenges associated with the translation of research from bench to patient. I gleaned that, despite general discussion idealizing “the” cure to cancer, there is no single cure for cancer, because cancer is a sneaky, evolving, scrappy sort of thing; it ducks punches delivered from the smartest scientists in the world; it’s like the mythical Hydra, in which the hero Hercules cuts off one head only for three more to grow in its place.

But the fight continues because occasionally, we make a little step in the right direction. Eventually, Hercules realized that he would never win if the heads could keep coming back; he had to stop the heads from proliferating. As Hercules beheaded the Hydra, his nephew used a torch to cauterize the headless tendons of the neck; and finally, all heads were gone, and the Hydra was defeated. There is a similar principle at work with an approach called “differentiation therapy”: traditional chemotherapy or radiation therapy will kill the cancer cells, but differentiation therapy causes the cells to progress from stem cells to terminal cells, so that they lose their intrinsic dividing ability.

Differentiation therapy was developed to extraordinary success for the treatment of acute promyelocytic leukemia (APL), a cancer of the blood in which immature blast cells crowd out normal cells in the marrow. A combination of all-trans retinoic acid (ATRA) and arsenic trioxide (ATO) causes the leukemic blast cells to differentiate into blood cells, and it is miraculously effective: 90% of patients experience complete remission. Unfortunately, this treatment does not work for all leukemias; but still, how might it work for other cancers? As heterogenous as they are, cancers follow fundamental rules: one cell undergoes a malignant transformation to resemble a proliferative stem cell, dividing excessively with no way to stop, eventually forming masses that invade organs and tissues.

In my readings, I found a fascinating paper by Hu et al. (2016), in which they determined a multi-target neuronal differentiation (MTND) therapeutic cocktail that could induce the terminal differentiation of a subtype of glioma (brain cancer) cells. From this, I designed a hypothetical preclinical study to elaborate on the effects of this treatment in mouse model. Does MTND have effects on another subtype of glioma? Furthermore, does MTND treatment extend survival in mouse models, and does it perform better than control and gold standard chemotherapy? Therein was my proposal – my little step in the (hypothesized) right direction – that I’m proud to say was praised as one of the best submissions in the class.

It was one of my favourite courses from my undergraduate experience, as you may have gathered from the sheer length of this preamble. I will stop myself here to let you read my actual work: please scroll down!