# Role of RFamide-Related Peptide 3 in Glioblastoma

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## Abstract

Glioblastoma (GBM), classified by World Health Organisation as a grade IV glioma, is a group of highly heterogeneous and aggressive neoplasms infamous for being a huge thorn on the side of GBM treatment avenues. Nothing demonstrates the need for an improved treatment regimen better than the fact that very few patients survive two years post-diagnosis, and not even one-tenth of the patients survive five years post-diagnosis. However, a breakthrough in improving the survival and quality of life in GBM patients has yet to surface. Neuropeptides have little significance in the grand scheme of GBM treatments, which could be an area that could use more study and research. Reproductive neuropeptides such as gonadotropin releasing hormone (GnRH) and kisspeptin have been found to induce apoptosis, reduce proliferation, and suppress metastasis in various cancers via their respective receptors. Gonadotropin inhibitory hormone (GnIH), also known as RFamide-related peptide 3 (RFRP-3) is a prominent figure in the regulation of GnRH release. Very little is known about the function of RFRP-3 in cancers. To that end, we aimed to investigate any potential therapeutic role of RFRP-3 in GBMs exerted via its specific receptor, namely GPR147. First and foremost, we aimed to establish the presence of RFRP-3's specific GPR147 via quantitative PCR (Q-PCR) and Western Blotting, where we discovered the receptor's presence, but only in GBM cells resistant to temozolomide (TMZ), a component of the standard modality therapy against GBM. The active status of GPR147 which binds to $G_{\alpha i}$ was determined via cAMP assays. We sought to determine the effect of RFRP-3 on the proliferation and apoptosis of GBM cells via MTT assays and flow cytometry with the presence of active RFRP-3 receptor. Unfortunately, our experiments showed a lack of involvement of RFRP-3 in both proliferation and apoptosis in GBM cells. With the absence of RFRP-3 receptor in chemosensitive GBM cells, we postulated the ability of RFRP-3 to affect the expression of $O^6$-methylguanine-DNA-methyltransferase (MGMT) gene in GBM which is the central blockade in TMZ treatment. To achieve that goal, we measured the expression of MGMT gene after concomitant treatments of RFRP-3 and TMZ via Q-PCR. Despite early promising results, the concomitant treatments failed to show any meaningful increase in cell death, highlighting the lack of modulation of MGMT gene expression by RFRP-3. Taken together, the results suggest the lack of a therapeutic role assumed by RFRP-3 in either proliferation, apoptosis, or the regulation of MGMT expression.

## Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Signature: 

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## Contents

# 1. Introduction

## 1.1 Glioma

The vast amount of tumour types in the brain and central nervous system (CNS) makes glioma, the most prevalent brain tumour type amongst adults stick out like a sore thumb, making up 81% of all malignant brain tumours (1). Classification of brain tumours is done based on the tumour's presumed cell of origin. Glioma is a brain tumour that originates from the glial cells of the CNS. Glial cells are supporting cells which provide oxygen and nutrients to the neurons and are by far the most populated cell type in the CNS. Unlike most types of cancer, gliomas are not staged and are instead classified into different grades by World Health Organisation (WHO) based on their histopathological characteristics and molecular genetics (2). Glioma is a broad classification of a group of tumours that includes astrocytoma (including glioblastoma), oligodendroglioma, ependydoma, oligoastrocytoma, malignant glioma and a few rare histologies (3). Table 1 shows the classification of the various glioma tumours and their characteristics (4, 5).

*Table 1: Characteristics of various glioma tumours. Information on the various glioma tumours was taken and modified from Rich et al. and the statistical report from CBTRUS (5, 6).*

| Grade | Histology | Median Incidence Age | 5 Year Relative Survival Rates | Characteristics |
|---|---|---|---|---|
| I | Pilocytic Astrocytomas | 12 | 94.1% | Relatively benign |
| II | Diffuse Astrocytomas<br>Oligodendroglioma<br>Oligoastrocytoma | 48<br>43<br>42 | 50.4%<br>81.6%<br>63.7% | Well differentiated cells that diffusely invade normal brain structures. |
| III | Anaplastic Oligodendrogliomas<br>Anaplastic Astrocytoma<br>Anaplastic Oligoastrocytoma | 50<br>48<br>42 | 57.6%<br>30%<br>63.7% | Has a more aggressive growth rate than grade II and tends to invade neighbouring tissues |
| IV | Glioblastoma | 65 (Primary)<br>45 (Secondary) | 5.6% | Highly invasive in the brain<br>Able to develop de novo to form primary glioblastoma or from low-grade tumours to form secondary glioblastoma |

A quick delve into the demographic of glioma patients will reveal that the incidence rate and age vary significantly by histologic type and the grade. According to a statistical report done by the Central Brain Tumor Registry of the United States (CBTRUS), grade IV glioblastoma was the most common glioma tumour amongst adults in the US, accounting for 56.6% of all gliomas, eclipsing all the other glioma subtypes which accounted for no more than 7.4% of all gliomas (6). The incidence age of glioma differs according to the grade of glioma, with pilocytic astrocytoma (grade I) being most common amongst children in the 0 – 9 age group, oligodendroglioma and oligodendroastrocytoma patients are all commonly found amongst adult in the 35 – 44 age group while glioblastoma is found to have a peak in the 75 – 84 age group (6, 7). The increased incidence rate with age especially amongst the elderly is postulated to be associated with deficits in normal immunosurveillance and progressive immunological changes throughout the body that comes with age (8). The incidence rate of glioma in the United States has been relatively stable across the years, with minimal changes in the incidence rates for all the age groups (6).

Survival of glioma patients also differs by grade across all glioma subtypes. Pilocytic astrocytoma patients have the highest survival rate, with 94.1% of the patients surviving five years post-diagnosis (6). The five-year relative survival rates decrease as the grade ascends, with glioblastoma patients displaying the most inferior overall survival at a measly 5.6% (6). Age significantly affects the survival rate of glioma patients, and the effect is most pronounced in glioblastoma patients, posting the highest age group in terms of peak incidence age as well as the lowest survival rate. Glioma patients with an oligodendroglial component often have a higher chance of survival as opposed to those with an astrocytic component, regardless of the glioma grade (6). Established molecular markers of gliomas include MGMT methylation, the mutation status of isocitrate dehydrogenase (IDH) gene 1 and 2, concomitant loss of 1p/19q genes and suppression of the tumour suppressor p53 gene and point mutations in the telomerase reverse transcriptase (TERT) gene promoter (9).

Multiple risk factors have been postulated to contribute to the development of glioma, with one of them being constitutive genetic risks. While underlying familial disorders rarely influence the development of glioma, there are exceptions for several rare inherited syndromes. Numerous monogenic Mendelian disorders such as Li-Fraumeni, tuberous sclerosis, Rubinstein-Taybi and enchondromatosis disorders (Ollier disease and Maffuci syndrome) are linked to the development of glioma (4). However, it has to be taken into account that these familiar cancer syndromes only account for a small population of adults afflicted with glioma (7). Genome-wide association studies (GWAS)

have provided a polygenic model that better explains the association between genetic risks and gliomagenesis. Several GWAS of glioma patients conducted have identified eight independently significant germline DNA single nucleotide polymorphisms (SNP) as glioma risk alleles, including variants in the TERT allele, CCDC26 allele, CDKN2B allele, PHLDB1 allele, TP53 allele, RTEL1 allele and two variants in the epidermal growth factor receptor (EGFR) allele (10-14). The presence of inherited variation of CDKN2B on chromosome 9p21, alterations in the TERT allele further corresponds to increased risk of high-grade gliomas (10).

Allergies and atopic diseases such as asthma, hay fever, eczema, and food allergies have often coincided with a decrease in glioma risk. An epidemiological meta-analysis actually revealed that allergies reduced the risk of glioma by nearly 40% (15). The inverse relationship between allergic conditions and glioma risk is predicated on the increased activity of the immune system that leads to enhanced tumour surveillance and killing of tumour cells. One of the biomarkers of allergy, immunoglobulin E, was found to be present at lower levels in glioma patients according to several studies (16-19), which divulged an inverse relationship between the total levels of IgE and the risk of glioma.

Ionising radiation was also touted as a possible environmental risk factor for gliomagenesis, where data from atomic bomb survivors coincided with an increase in glioma risk in a linear dose-response (20). Survivors from previous cancer treatments with high exposure to radiation treatments also conveyed increased odds of succumbing to glioma (21, 22). With CT scanning being a more prevalent medical procedure, the cumulative effects of radiation from CT scanning are also becoming a concern, with studies evaluating and pointing out the increased risks of glioma that comes with cumulative exposures to the head (23, 24).

Studies concerning the lifestyle and diet choices and the risks of gliomas have yielded mixed results. N-nitroso compounds are potentially carcinogenic, mainly due to the presence of nitrite and nitrate and are mostly found in processed foods such as cured meats (4). Several meta-analyses have attempted to investigate any association between red or processed meat with mixed results. Wang *et al.* looked at nine studies that included 2264 adult glioma cases and concluded that dietary nitrite and nitrate had no consequences on the risk of afflicting glioma (25). Wei and his colleagues found a correlation between processed meat consumption and increased risk of glioma, but only in case-control studies, not in cohort studies (26). Saneei *et al.* on the other hand, found a positive association

between unprocessed red meat and increased glioma risk, again based entirely on case-control studies. After considering the total energy intake for each study, no association was found between the consumption of processed meat and glioma risk (27). Conclusions regarding this issue are hard to come by since other genetic and environmental variables could skew results and case-control studies have inherent limitations that include recall or selection bias (26). Occupational chemical exposures have also been studied quite extensively for years, with nothing but mixed results as well (7).

## 1.2 Glioblastoma

Glioblastoma multiforme, categorised as the highest grade of GBM (grade IV) by WHO, is the most aggressive and the most common of all malignant brain tumours, with GBM alone accounting for a little under half (47.7%) of all malignant brain tumours in the United States (6). GBMs are histologically characterised as an astrocytic tumour, composed of mostly poorly differentiated astrocytic lesions. As its name implies, GBMs have a multiforme nature, displaying regions of necrosis, nuclear atypia, aberrant mitotic activity, and microvascular proliferation which are typical histologic criteria for high-grade infiltrating astrocytic tumour. GBMs are also further divided into primary and secondary GBMs. At a population level, primary GBMs are the overwhelmingly prevalent GBM amongst the two, with 90% of all GBM cases being reported as primary GBMs (28). Primary GBMs are also termed *de novo* GBMs as these tumours are diagnosed as full-blown tumours with no clinical, radiological, or histopathological evidence of a less-malignant precursor lesion. However, like most human neoplasms, the development of primary GBM results from the acquisition of multiple genetic alterations. Secondary GBMs by contrast, develop slowly through the progression from low-grade diffuse astrocytoma or anaplastic astrocytoma and thus accrue a different set of genetic modification than those of primary GBMs. Due to the development of the tumour through distinct genetic pathways and the lack of overlapping genetic alterations between primary and secondary GBMs, they are considered to be distinct disease entities (29). The rarity of secondary GBMs is especially highlighted considering the combined incidence of diffuse and anaplastic astrocytoma is approximately two to three times higher than that of secondary GBMs (30). One of the reasons for its rarity may be partially explained by the fact progression into GBM from diffuse astrocytoma and anaplastic astrocytoma rarely occurs, considering both the grade II and grade III gliomas do not boast a high survival rate.

Up till the early 2000s, GBMs were diagnosed primarily by their histopathological characteristics, and thus molecular markers were less defined to predict the outcome of treatments across a broader population of patients. Further prognostic classification serves to provide valuable utility in offering

prognostic value that better predicts the outcomes of GBM cases. The availability of high-throughput genomic platforms for mRNA expression profiling led to researchers attempting to divide GBMs into subclasses based on gene expression signatures. In 2006, Phillips *et al.* used K-means clustering to analyse the different gene expression signatures associated with clinical outcome and delineated said signatures into three different subtypes for high-grade gliomas, namely proneural, mesenchymal and proliferative (31). The comprehensive catalogue of genomic abnormalities promoting GBM development detailing the genomic changes in a large GBM cohort performed by The Cancer Genome Atlas (TCGA) allowed Verhaak *et al.* to further classify GBMs into four different molecular subclasses (32, 33). Integrating the data obtained from TCGA that involved 206 patient samples, they were able to identify gene expression signatures that correlate to four different subtypes, proneural, neural, classical and mesenchymal (32). The proneural subtype is commonly associated with secondary GBMs and thus corresponds to patients with younger age and better survival probabilities, platelet-derived growth factor alpha (PDGFRA) abnormalities, IDH1 and TP53 mutations. The neural subtype included the expression of neuron markers, and their expression patterns are similar to normal brain tissues. Common genetic aberrations seen in GBMs such as harbouring chromosome 7 amplification (suggesting EGFR amplification[34]), chromosome 10 deletions (deletion of tumour suppressor genes such as phosphatase tensin homolog gene (PTEN), HNPRF, CXXCC etc.[34]), EGFR amplification and homozygous deletion of the *Ink4a/ARF* locus forms the constellation which defines the classical subtype. The identity of mesenchymal subtype, on the other hand, is defined by the combination of high CHI3L1 and MET expression and alterations to the neurofibromatosis type I (NF1) gene (32).

Overall age-adjusted incidence rates (adjusted to the national population of each respective study) have been used to provide a consistent parameter to allow ease of comparison between different respective studies in contrasting populations and countries. Age-adjusted incidence rates for all gliomas range from 4.67 to 5.73 per 100000 persons (7). As previously mentioned, GBMs are the most common and the deadliest glioma subtypes amongst adults, with the age-adjusted incidence of GBMs ranging from 2.9 to 4.67 per 100000 persons (Table 2). In the United States, GBMs was far and away the most prevalent tumour amongst glioma subtypes with an incidence rate of 3.03 per 100000 persons, followed by diffuse astrocytoma (0.44/100000 persons) (6). GBMs are most often diagnosed amongst the elderly and are much rarer amongst children and teens, with GBMs accounting for only 3.1% of all brain and other CNS tumours reported in the 0 – 19 years age group. Incidence of GBMs increases with age, peaking in individuals ranging from 75 – 84 years (6). There is a difference however, in the age distribution of patients with primary and secondary GBMs. Primary GBMs are more frequent amongst the elderly, with a recorded median age of 65, but secondary GBMs develop in younger

patients with a median age of approximately 45 years (6, 29). GBMs are also generally more prevalent amongst males than females, with a population-based male to female ratio ranging from 1.28 to 1.32 (35, 36). In contrast, the male predominance when it comes to diffuse astrocytoma is slightly weaker, with the ratio coming in at 1.17 (36). Considering secondary GBMs develop from lower-grade gliomas, typically from diffuse astrocytoma, a similar gender ratio is also observed in secondary GBMs. Secondary GBMs had a male to female ratio of 1.12 according to a population-based study (35). This finding was consistent amongst several hospital-based studies as well, which showed a similar trend of slightly lower male to female ratios in the case of secondary glioblastomas (37, 38).

*Table 2: Age-adjusted incidence rates of glioblastoma per 100000 persons, by country/region (all ages)*

| *Region* | *Years* | *Rate (per 100000 persons)* | *95% CI* |
|---|---|---|---|
| *Australia* [39] | 2000 – 2008 | 3.96 | 0.4 – 4.6 |
| *United States* [6] | 2011 – 2015 | 3.21 | 3.18 – 3.23 |
| *Finland* [40] | 2000 - 2013 | 2.9 | - |
| *England* [41] | 2007 - 2011 | 4.64 | 4.56 – 4.73 |
| *Greece* [42] | 2005 – 2007 | 3.69 | - |
| *Korea* [43] | 2005 | 0.59 | - |

The advancement in genomic technology has facilitated the identification of various molecular markers associated with GBM. This has allowed researchers to categorise various GBMs into different subtypes as well as allowing clinicians to identify more suitable therapies to better cater to a patient's needs. GBMs exhibit a variety of significant genetic heterogeneity that carry precious prognostic values. The reason primary and secondary GBMs are considered to be different disease entities is down to the fact that both GBMs have distinct prognostic molecular markers. Primary GBMs often show EGFR overexpression, PTEN mutations, heterozygous loss of chromosome 10, chromosome 7 amplification, TERT promoter mutations, MDM2 amplification and lack of IDH1 mutations. Secondary GBMs contrarily, display genetic alterations in α thalassemia/mental retardation syndrome X-linked (ATRX) and IDH1 mutations. GBM patients also display various clinical outcomes across the age groups as a consequence of the complex interplay between age and genetic heterogeneities (44). Patients aged 40 years and below are usually associated with a much more favourable prognosis and more prolonged survival when compared to patients aged 40 years and above (45).

GBMs have largely evaded most therapeutic approaches beset upon it, and thus GBMs are notorious for its low relative survival estimates. Very few patients survive two years post-diagnosis, with GBM patients that survive five years post-diagnosis being few and far between (only 5% - 5.6% of the patients) (6, 46). Chandler *et al.* revealed in their population-based study that the peak mortality occurs in the first quarter of the second year post-diagnosis, with the rate plummeting to half of the peak mortality by the second quarter (46). It was also proposed that those who have a prolonged disease-free spell after the initial diagnosis while receiving the standard GBM therapy have a higher probability of longstanding survival, typically those who survive two years post-diagnosis (46, 47). A detailed analysis by Krex *et al.* of 55 primary GBM long-term survivors recruited from 6 clinical centres in Germany also underlined that GBM long-term survival is more probable at a younger age (48). The median survival of primary and secondary GBM patients are also different, with secondary GBM patients having a significantly longer median survival at 7.8 months compared to primary GBM patients at 4.7 months. However, this difference can be traced back to secondary GBM patients' younger age group. Multivariate analyses that took age adjustments into accounts revealed that the survival rates of primary and secondary GBM patients do not differ significantly (35, 49). In Krex *et al.*'s analysis of the 55 GBM patients in Germany, long-term survival in GBM patients was not only associated with younger age but also seemed to favour the females (48). This was consistent with Trifilletti *et al.* and Ostrom *et al.*'s findings where they disclosed a survival advantage amongst female GBM patients with a far larger sample size, looking into 27865 patients and 5372 GBM cases respectively (50, 51).

Upon analysing the transcriptome of GBM patients of the different sexes, Wei *et al.* found a few insights that distinguished male and female GBM at a molecular level, identifying the presence of sex-specific clusters that are distinguished by gene expression that provided a survival advantage towards female GBM patients (52). The fascinating work done by their group revealed a cluster of genes expressed explicitly by each respective sex that corresponded to higher overall survival. It was found that the female-specific cluster exhibiting a median overall survival of 1172 days while the male-specific cluster exhibited a median overall survival of 620 days. They also revealed that the two different GBM subtype did not affect the survival advantage offered by the female's gene cluster but secondary GBMs stratified survival in the male's gene cluster cases. Their study provided a solid foundation to suggest that sex-specific analysis can give rise to a more precise GBM molecular subtyping. They further suggested that by tailoring treatment according to gender, improved outcomes in terms of patient survival might be brought about (52).

### 1.2.1 Therapeutic Avenues for GBM

As it stands, standard therapy against GBM, known colloquially as the Stupp protocol, involves a combined modality therapy including maximal safe resection followed by radiotherapy and treatment with TMZ. Standard therapy against GBM that is still in use was established back in 2004 when adjuvant temozolomide treatment in conjunction with radiotherapy was performed in a randomised phase III trial conducted by the European Organisation for Research and Treatment of Cancer (EORTC) which increased the median survival from 12.1 months to 14.6 months (53). The result of the clinical trial prompted the approval of TMZ from the Food and Drug Administration (FDA) as a legitimate therapeutic agent against GBM (53). The combined modality treatment consistently displayed a significant increase in overall survival across three different prognostic EORTC recursive partitioning analysis classes when compared to radiotherapy alone (54). Furthermore, the survival benefit of the Stupp protocol also translated into a population-based cohort, with patients diagnosed in 2005 – 2006 (the year Stupp protocol was established) boasting superior survival benefits compared to those diagnosed in 2000 - 2001 (55). Patients diagnosed in 2005 – 2006 was shown to have an increased 11% in median and 2-year relative survival over patients diagnosed in 2000 – 2001 (55). However, the median overall survival of GBM patients remains between 10 – 12 months, with 75% of GBM patients succumbing to the disease within two years of initial diagnosis (56).

**Radiotherapy**

Global interest in improving the outcome of GBM patients never wavered despite the establishment of the Stupp protocol. The source of radiotherapy is still under intense scrutiny for further improvements despite the reliability of the Stupp protocol. Particle therapies have been touted to replace traditional photon therapies due to the advantageous physical properties inherent in high energy protons and carbon ions such as proton beam therapy (PBT) and carbon ion radiotherapy (CIRT). The signature Bragg peak pattern exhibited by high energy protons and carbon ions provides them with superiority in the form of energy deposition at the target while theoretically allowing higher doses to be delivered to the target specifically all the while limiting energy exposure to adjacent normal tissues (57, 58). While PBT and CIRT have shown promising signs in their respective clinical trials (59-61), treatments such as CIRT come with a high financial burden, with only a few centres around the world capable of delivering CIRT (58, 62).

Ongoing attempts are also being made to improve the efficacy of proton therapies. Radiotherapies rely on DNA damage to inflict cytotoxic effect, and DNA repair pathways are a bane of radiotherapy

efficacy. Poly(ADP-ribose) polymerases (PARPs) are highly active in the base excision repair pathway, recruiting proteins and facilitating the repair of DNA adducts. The propensity of reaching therapeutic levels *in situ* prompted investigations into PARP inhibitors such as olaparib, veliparib and pamiparib. The efficacy of olaparib and veliparib in conjunction with the standard of care in GBM patients are being evaluated in separate clinical trials, which was well tolerated amongst patients but only yielded slight survival benefits (63-65). Radiosensitisers that target the homologous recombination repair and non-homologous end-joining that repair double-stranded breaks (DSBs) such as CC115, VX-984 and KU-60019 have shown promising potential as candidates and are all under preclinical or early clinical investigations (62, 66-69).

*Table 3: Ongoing (recruiting, yet to recruit, active) phase I, II or III clinical trials for radiotherapies and radiosensitisers*

| Category | Target | Therapy | Disease | Trials | Phase |
|---|---|---|---|---|---|
| Particle Therapies | - | PBT | Newly Diagnosed | NCT02179086 | II |
| | | | Recurrent | NCT01730950 | II |
| Radiosensitizers | PARP | Olaparib | Recurrent | NCT02974621 | II |
| | | | Recurrent | NCT03212274 | II |
| | | Veliparib | Newly Diagnosed | NCT02152982 | II/III |
| | | | Newly Diagnosed | NCT03581292 | II |
| | | Pamiparib | Newly Diagnosed | NCT03150862 | I/II |
| | DNA-PKcs | CC-115 | Recurrent | NCT02977780 | II |
| | ATM | AZD1390 | Recurrent | NCT03423628 | I |

*Keywords:* ***PBT*** *proton beam therapy,* ***PARP*** *poly(ADP-ribose) polymerase,* ***DNA-PKcs*** *DNA protein kinase catalytic subunit,* ***ATM*** *ataxia telangiectasia mutated protein*

**Targeted Therapies**

According to the Cancer Genome Atlas Research, receptor tyrosine kinase (RTK)/Ras/phosphatidylinositol 3-kinase (PI3K), p53 and retinoblastoma (Rb) signalling pathways are the three pathways that are most commonly subjected to alterations and mutations in the development of GBM. EGFR-targeted therapies such as erlotinib and gefitinib that act as small molecule kinase inhibitors were first investigated as a way to circumvent the amplification of the EGFR gene (70, 71). However, the effects of erlotinib and geftinib were less than desirable, displaying little effects in clinical trials, with the downstream components remaining constitutively active (72, 73). Combined therapy of TMZ and radiotherapy with erlotinib also showed minimal survival benefits (74). Several measures were also taken to inhibit the RTK pathway by suppressing the downstream

PI3K/AKT/mTOR signalling pathway. Attempts to inhibit PI3K was attempted using PX-866 and BKM120 was met with promising initial results but unfortunately said promise was not replicated in its ensuing clinical trials (75-77). Rapamycin, temsirolimus and everolimus are examples of mTOR inhibitors which received FDA approval used in the treatment of various solid cancers, although it exhibited minimal clinical activity in GBM patients (70).

p53 is a tumour suppressor protein that is involved in complex carcinogenesis signalling pathways that involves regulating various responses such as cell cycle arrests in the $G_1/G_2$ phase of the cell cycle as well as cell apoptosis upon DNA damage and is altered in most cancers including GBM (78). Disruption of p53 can come from the amplification of MDM2, an E3 ubiquitin ligase that acts as a negative regulator of p53 expression found in approximately 10% of GBM, specifically in primary GBMs that lack any p53 mutations (33, 79). Nutlin-3 is a well-studied cis-imidazoline MDM2 antagonist that binds to MDM2 by mimicking crucial residues in the transactivation domain of p53 (80). Therapeutic strategies targeting p53 involved molecular alteration using nutlin-based drugs to inhibit MDM2. Preclinical trials have demonstrated nutlin-based drugs' (RG7112 and ISA27) anti-tumour efficacy in MDM2 amplified GBM models (81, 82).

Rb1 protein maintains the cell cycle by acting as the checkpoint of the progression from $G_1$ phase into the S-phase. One of the most common alterations in the Rb signalling pathway is the amplification of CDK4 and CDK6, leading to the development of palbociclib, which acts as a highly specific CDK4/6 inhibitor (83). Results from Michaud *et al.* indicated the efficacy of palbociclib suppressing the growth of GBM both *in vivo* and *in vitro* GBM models, however, the BBB remains a limiting factor of the delivery of palbociclib to invasive GBM cells (83, 84). A recently published phase II trial using palbociclib demonstrated tumour concentrations of palbociclib that were thought to be biologically significant, but the trial was halted early due to futility and lack of efficacy as 95% of the patients progressed within six months of treatment initiation (85).

VEGF is another target specifically earmarked as a therapeutic target for GBM. Angiogenesis is rampant amongst cancers, and GBM is amongst the most angiogenic of all malignancies (86). For GBM metastasis to progress, it is vital that new blood vessels are formed. VEGF is the prominent angiogenic factor facilitating the process, and its activation can either be dependent on hypoxia or independent from hypoxia. Bevacizumab is a monoclonal antibody used in the treatment of various cancers, including GBMs. Angiogenesis was inhibited in preclinical studies, and it was also found to augment

the cytotoxicity of either radiation or temozolomide *in vivo* (87-89), but it failed to confer any significant survival advantage in several phase III trials even when administered in combination with the Stupp protocol (90, 91). In addition to targeting the ligand in the form of VEGF, attempts have also been made to inhibit VEGF receptors which are tyrosine kinase receptors. Examples of VEGF-R inhibitors include cediranib, sunitinib and pazopanib, which has shown negligible survival benefits in multiple clinical trials (92-94).

*Table 4: Ongoing (recruiting, yet to recruit, active) phase II or III clinical trials for targeted therapies*

| Target | Agent | Disease | Trials | Phase |
|---|---|---|---|---|
| EGFR | GC1118 | Recurrent | NCT03618667 | II |
| | ABT-414 | Newly Diagnosed | NCT02573324 | II/III |
| | Osimertinib | Recurrent | NCT03732352 | II |
| | Neratinib | Newly Diagnosed | NCT02977780 | II |
| PI3K/mTOR | Paxalisib | Newly Diagnosed | NCT03522298 | II |
| mTOR | Sirolimus | Newly Diagnosed | NCT03463265 | II |
| | | Recurrent | NCT03463265 | II |
| | Everolimus | Newly Diagnosed | NCT01062399 | I/II |
| | | Recurrent | NCT01434602 | I/II |
| CDK 4/6 | Palbociclib | Newly Diagnosed | NCT03158389 | I/II |
| | Abemaciclib | Recurrent | NCT02981940 | II |
| VEGF-R | Cediranib | Newly Diagnosed | NCT01062425 | II |
| | | Recurrent | NCT02974621 | II |
| | TTAC-0001 | Recurrent | NCT03856099 | II |
| | Lenvatinib | Recurrent | NCT03797326 | II |
| | Anlotinib | Recurrent | NCT04004975 | II |
| | Lenvatinib | Recurrent | NCT03797326 | II |
| VEGF-R/TIE2/RAF1/KIT/RET/BRAF | Regorafenib | Newly Diagnosed | NCT03970447 | II/III |
| | | Recurrent | NCT03970447 | II/III |
| | | Recurrent | NCT04051606 | II |
| BRAFV600E/MEK | Dabrafenib + Trametinib | Newly Diagnosed | NCT03919071 | II |
| | | Recurrent | NCT02684058 | II |
| B2-GP1 | Bavituximab | Newly Diagnosed | NCT03139916 | II |
| Ang | Trebananib | Recurrent | NCT01609790 | II |
| BTK | Acalabrutinib | Recurrent | NCT02586857 | I/II |
| DRD2/ClpP | ONC201 | Recurrent | NCT02525692 | II |
| HIF-2α | PT2385 | Recurrent | NCT03216499 | II |
| XPO1 | Selinexor | Newly Diagnosed | NCT04421378 | I/II |
| | | Recurrent | NCT04421378 | I/II |
| GSK-3β | Belinostat | Newly Diagnosed | NCT02137759 | II |

*Keywords:* ***EGFR*** *epidermal growth factor receptor,* ***PI3K*** *phosphatidyl-inositol-3 kinase,* ***mTOR*** *mammalian target of rapamycin,* ***CDK*** *cyclin-dependant kinase,* ***VEGF-R*** *vascular endothelial growth factor receptor,* ***TIE2*** *angiopoietin-1 receptor,* ***RAF1****,* ***β2-gp1*** *beta-2 glycoprotein 1,* ***Ang*** *angiopoietin,* ***BTK*** *Bruton's tyrosine kinase,* ***DRD2*** *dopamine receptor D2,* ***ClpP*** *caseinolytic protease P,* ***HIF-2α*** *hypoxia inducible factor-2 alpha,* ***XPO1*** *exportin 1,* ***GSK-3β*** *glycogen synthase 3 beta*

The challenge in the pursuit of a therapeutic avenue as mentioned earlier is the low median survival and relapses within GBM patients. Standard of care for GBM patients remains unaltered and ineffective, and despite ongoing efforts in incorporating targeted therapies and novel radiotherapies, a major breakthrough has yet to be unveiled. This is most likely caused by the fact that malignant glioblastomas are highly heterogenous genetically (33), which leads to the development of genetically different subtypes within a tumour. The aggressive nature and multifaceted resistance mechanisms of malignant GBM also involve multiple signalling pathways which are sometimes interconnected with one another (70, 95). The relapse of GBM could also be caused by glioblastoma stem cells (GSCs) which are cells within glioma that are endowed with stem cell properties. It is suggested that genetic heterogeneity exists within GSCs, which can give rise to genetically different populations within a tumour (96). Nonetheless, the synergistic effect of combining the standard of care with targeted therapies and immunotherapies may still prove crucial in the fight against GBMs. Therefore, a detailed molecular characterisation of GBM is not only needed, but there is also a demand to identify more molecular targets that can either inhibit signalling pathways involved in tumour progression or activate signalling pathways that are involved in tumour suppression (70, 95).

## 1.3 Temozolomide

As mentioned earlier, chemotherapy with TMZ is one of the standard therapies for GBM. TMZ is widely used in GBM treatment as it introduces DNA lesion to GBM cells which ultimately leads to apoptosis. Before the approval of TMZ by the FDA, nitrosoureas-based drugs were administered in brain cancer chemotherapy (97). N-methyl-N-nitrosourea (MNU) was the first methylating agent used in brain cancer chemotherapy. MNU managed to exert strong neurotropic carcinogenic activities in rats, and the neurotropic carcinogenicity of MNU was attributed to the development of $O^6$-methylguanine ($O^6$-meG) that were left untreated (98). However, MNU tends to display some severe carcinogenic effects due to its ability to decompose spontaneously, which leads to unwanted DNA alkylation within minutes (97). This led to the discovery of procarbazine, carmustine (BCNU) dacarbazine (DTIC) which alleviated fears of strong systemic side effects. Streptocotozine was also developed, which was MNU coupled to glucose and was highly useful for tumours expressing glucose transporters (97). However, these nitrosoureas-based agents not only required metabolic activation but also failed to produce significant survival benefits when compared to radiotherapy alone in randomised phase III trials (53). Chang *et al.* found that median survival of patients was not improved upon by nitrosoureas agents such as BCNU (8.1 months) and DTIC (8.9 months) compared to patients receiving only radiotherapy (8.7 months) (99). On that premise, Green *et al.* found that adjuvant BCNU treatment with radiotherapy yielded similar survival curves, providing little survival benefits over patients that only received radiotherapies (100). This garnered widespread interest for TMZ, a triazene derivative that can modify the nitrogen atoms of the aromatic rings and the oxygen groups of DNAs via an $S_N1$ nucleophilic reaction, first discovered in 1984 by Stevens and his co-workers (101, 102). TMZ replaced the nitrosourea-based drugs previously administered as the standard chemotherapy after the clinical trials conducted by the EORTC.

TMZ causes cell cycle arrest particularly at the $G_2$/M phase, facilitating the eventual death of the cells (103). TMZ has been proven to improve the prognosis of GBM patients owing to the fact that its lipophilic nature allows TMZ to cross the BBB efficiently. Unlike the aforementioned nitrosoureas-based agents that require metabolic activation, TMZ is activated in a non-enzymatic, pH-dependent chemical degradation process with the added benefit of exhibiting only mild and reversible myelosuppression (104). Commonly, 150 or 200mg/$m^2$ of TMZ is administered daily for five days at a maximum tolerated dose of 1000mg/$m^2$ in each 28-days cycle (105). The dosage standard established by the phase 1 and II clinical trials conducted by the Cancer Research Campaign (CRC) demonstrated the negligible toxicity level of TMZ as well as its potential of improving the quality of life in glioma

patients (106-108). Common nonhematologic unfavourable effects include nausea, vomiting, headache, fatigue, and constipation. The severity posed by these adverse effects were nothing more than moderate, with nausea and vomiting easily controllable with standard antiemetics (104). Out of the 103 eligible patients in the phase II trial conducted by the CRC, the major side effect was myelosuppression, with 16 episodes of grade 4 lymphopenia and 7 episode of grade 4 thrombocytopenia (107). Nonetheless, TMZ treatments rarely resulted in the need for treatment discontinuation. In general, 150mg/$m^2$ of TMZ is administered daily for five days for patients who had previously undergone chemotherapies, with a planned increase to 200mg/$m^2$ per day if no major myelosuppression was observed on day 22 of the 28-day cycle. Previously untreated patients receiving chemotherapy treatment on the other hand typically receive 200mg/$m^2$ per day for five days. A phase I trial was also devised to evaluate the effectiveness of a continuous oral dosing schedule. A dose-dense schedule which involved administration of 75mg/$m^2$ of TMZ every day over a period of maximum 49 days resulted in twice as much exposure and this regimen allowed for the possibility of administering TMZ in conjunction with radiotherapy (109). Thus when TMZ is administered in conjunction with radiotherapy, the dose is reduced to 75mg/$m^2$ per day for the entire duration of radiotherapy up to a maximum of 49 days (53, 109). Patients are then given a rest for a month before given adjuvant TMZ treatment following the standard 5-day cycle every 28 days for up to 6 cycles (53).

### 1.3.1 Mechanism of Action

TMZ is a prodrug that belongs to the imidazotetrazine family, first synthesised in 1984 by Stevens and his co-workers, where they managed to showcase its anti-tumour property (102). TMZ is structurally similar to DTIC, containing an imidazole ring and is a member of the triazene subgroup (110). TMZ has an atomic mass of 194kDa, allowing it to diffuse effectively across the digestive tract and into the bloodstream, while its lipophilicity coupled with its small size allows it to penetrate the BBB (104). Direct evidence has been provided regarding TMZ's ability to penetrate the BBB via PET studies using $^{11}$C-labelled prodrug (111). Another advantage that works in favour of TMZ is the fact that TMZ can be administered orally, unlike its nitrosoureas-based predecessors that needed to be injected intravenously into the veins. Food intake does not have a huge impact on oral administration of TMZ, with adsorption reduced by a mere 9% (104). TMZ is rapidly absorbed into the bloodstream as studies have shown peak plasma concentration was achieved within 2 hours of administration, with more than 99% of the orally administered TMZ entering the blood plasma (112). The activation of TMZ is pH-dependent, and its robust acid stability allows it to remain stable in the stomach, further contributing to the drug's oral bioavailability. Unlike its predecessors like BCNU and procarbazine, TMZ

does not cross-link DNA strands, and thus are less damaging to the hematopoietic progenitor cells in the bone marrow (104). TMZ has a metabolic half-life of $t_{1/2}$ = 1.29h, which is ideal as a short half-life will lead to TMZ being inactivated before reaching its target while a long half-life could lead to TMZ being excreted without exerting any effects (113). In a pharmacokinetic study following the dose of 150 to 200mg/$m^2$ on a five-day schedule, it was found that TMZ is not only rapidly absorbed, but it was also rapidly eliminated, with all traces of administered TMZ disposed of within 8 hours (104). TMZ's pharmacokinetic properties allow TMZ to reduce the risk of causing any cumulative hematologic toxicity.

Studies involving pharmacokinetics of TMZ penetrating the CNS was done in non-human primate models (Rhesus monkeys). The study revealed that the penetration of TMZ into the cerebrospinal fluid (CSF) was substantial, with the CSF:plasma area under the concentration-time curve (AUC) ratio coming in at 33% (114). Ostermann *et al.* conducted the first pharmacokinetic study of the penetration of TMZ into the CNS in humans. The use of population pharmacokinetic approaches by assessing TMZ pharmacokinetics in plasma and CSF, along with its interindividual variability allowed the authors to conclude that the $AUC_{CSF}/AUC_{plasma}$ ratio in humans was 20% (115). They also explored the relationships between systemic or cerebral drug exposures and clinical outcomes and came to the conclusion that neither are better predictors than the cumulative dose alone for both efficacy and safety (115).

TMZ undergoes spontaneous alkaline hydrolysation into its active metabolite to give the open linear chain triazene 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) upon contact with the bloodstream's more basic pH environment (pH higher than 7). Further hydrolysation of MTIC produces 5-aminoimidazole-4-carboxamide (AIC) and an active methyldiazonium cation which reacts with the nucleophilic sites on DNA to facilitate the alkylation process (101, 103). MTIC is also the active metabolite of DTIC, however, while TMZ is hydrolysed into MTIC spontaneously, DTIC required initial active metabolism via hepatic demethylation from CYP450 oxidation and loss of formaldehyde (105, 113).

The active methyldiazonium cations methylate DNAs via an $S_N1$ nucleophilic reaction that follows the first order of kinetics which depends on the formation of electrophilic carbocations (116). TMZ mainly targets three alkylation sites, namely N7 position of guanine, N3 position of adenine and O6 position of guanine, producing $N^7$-methylguanine ($N^7$-meG), $N^3$-methyladenine ($N^3$-meA) and $O^6$-

methylguanine ($O^6$-meG) respectively (101). The most common alkylation site amongst the three positions is the N7 position of guanine, which is methylated 60 – 80% of the time, followed by N3 position of adenine and O6 position of guanine, which is methylated 10 – 20% and 5 – 10% respectively (101). Although O6 position of guanine is the least popular alkylation site, it is the leading position where TMZ confers its cytotoxicity, as alkylation of the O6 position produces O6-methylguanine (O6-MeG), causing the guanine to pair with thymine instead of a cytosine during DNA replication, and this can lead to apoptosis (103). Cytotoxicity induced by $O^6$-meG requires the presence of the mismatch repair (MMR) system. During DNA replication, MMR attempts to repair the incorrect $O^6$-meG: thymine pair via the proteins MSH2, MSH3 MSH6, MLH1 and PMS2. This process excises the thymine from the daughter strand, leaving $O^6$-meG on the template strand intact while creating a gap through exonuclease 1 (EXO1) activity (117). The error on the template strand remains, prompting thymine to be reinserted during the sealing of the gap, activating a futile and repetitive repair cycle which inevitably leads to DNA DSBs, activating the apoptotic pathway (113, 117). Despite the prevalent nature of $N^7$-meG, it remains the least cytotoxic adduct, since the methyl group of $N^7$-meG and $N^3$-meA is easily removed by the base excision repair (BER) system without damaging the DNA.

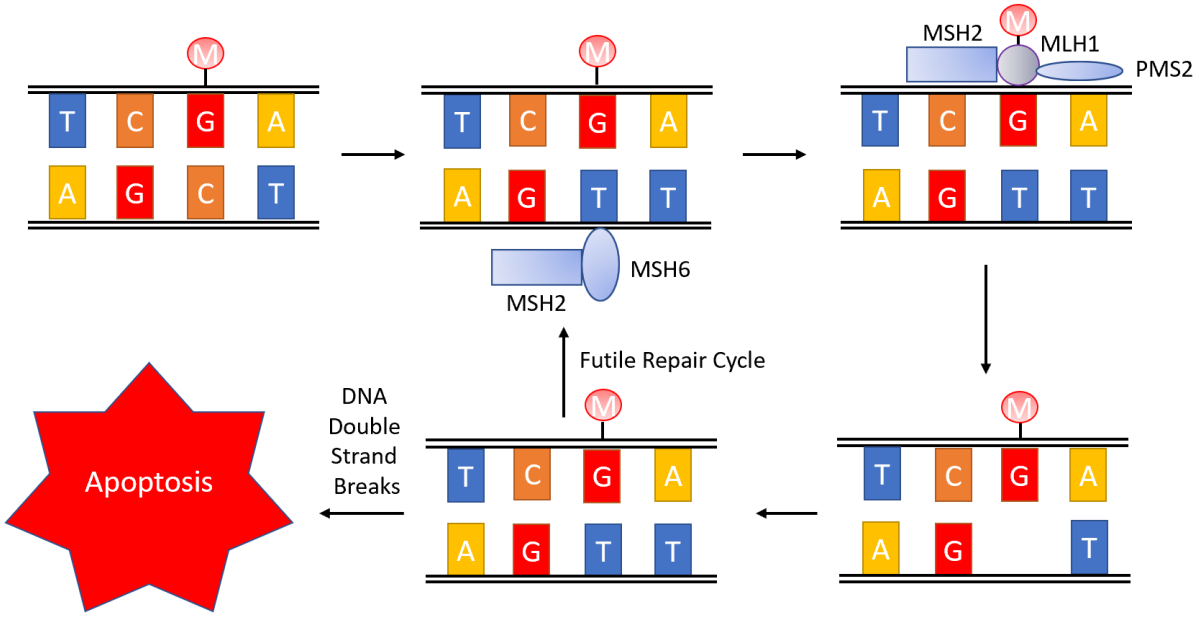



*Figure 1: Futile MMR repair cycle induced by the alkylation of $O^6$-methylguanine that eventually leads to apoptosis.*

### 1.3.2 Mechanism of TMZ Resistance

DNA lesions in humans are repaired by DNA repair mechanisms that have evolved to be robust and are prepared for any DNA damage that is caused either endogenously or by an exogenous source. The robustness of the DNA repair mechanisms arises from the redundancy of many different signalling pathways that can lead to the repair of DNA (118). A robust and accurate DNA repair system is essential in maintaining genomic stability, a characteristic notably missing in tumours. Thus, cancer cells are usually genetically or epigenetically modified to inhibit DNA repair. However, cancer cells often also rely on residual DNA repair mechanisms to help them survive DNA lesions caused by alkylating drugs such as TMZ (118).

With the major adducts of TMZ being $N^7$-meG and $N^3$-meA, BER can play a role in conferring TMZ resistance in GBM. The first point of contact by the BER is through N-methylpurine DNA glycosylase (MPG). The methylated bases are recognised and removed by MPG without cleaving the DNA, leaving an abasic site (AP site), which is subsequently repaired by AP endonuclease 1 (APE1). This results in an incision of the damaged strand followed by the formation of a 3'OH group and 5'deoxy-ribose phosphate (5'dRP) moieties in the gap (119). The gap is recognised by PARP1, PARP2 and poly(ADP-ribose) glycohydrolase (PARG) that recruit proteins to facilitate the repair, including a BER scaffold protein containing XRCC1 and DNA polymerase β (Polβ) (119). Polβ hydrolyses the 5'dRP, and a nucleotide is inserted in its place, followed by ligation by DNA Ligase IIIα and XRCC1 (119). Like other DNA repair systems, the efficiency of BER relies on the careful regulation of the expression of repair genes. Overexpression of MPG was found to have a sensitising effect in GBMs (120), with ablation of MPG expression demonstrating similar effects (121). Excessive expression of MPG actually produces DSBs due to overlapping repair patches (120). Therefore, inhibiting BER can be a legit alternative to sensitise GBM patients to TMZ (122).

One of the mechanisms of resistance for TMZ is the deficiency in the MMR system. As mentioned earlier, TMZ mainly confers its cytotoxicity via $O^6$-meG and the futile repair cycle of the MMR system. A comprehensive genome analysis done by The Cancer Genome Atlas (TCGA) revealed that 5 out of the 19 post-treatment GBMs had somatic MSH6 mutations (33), which indicated the resistance of recurrent GBM patients towards TMZ is down to the deficiency in the MMR system. Maxwell and his group declared that MMR deficiency does not correlate with TMZ resistance based on the absence of high microsatellite instability (MSI) (123). However, the result presented by Maxwell and co. was not conclusive as MSH6 mutation was associated with a low MSI phenotype. Yip's group also validated

TCGA's findings by comparing pre-treatment and post-treatment tumour tissues, which revealed post-treatment GBMs exhibited two distinct MSH6 somatic mutations (124). It was also found that the reduction in MLH1 and PMS2 expression confers TMZ resistance (125). Perazzoli's group determined the expression of the previously mentioned MMR proteins and discovered higher subunit expressions in TMZ sensitive cell lines and vice versa in TMZ resistant cell lines, further cementing the importance of MMR in conferring TMZ resistance in GBM (126).

However, MGMT remains one of the main culprits conferring TMZ resistance, which represents one of the central blockades towards more effective treatment of GBM patients. MGMT is part of the DNA repair system repertoire, an enzyme responsible for removing the methyl group from $O^6$-meG, transferring it to a cysteine residue (Cys145) within its active site in a suicidal reaction that leads to the ubiquitination and degradation of MGMT (118). This restores the structural integrity of $O^6$-meG, thus preventing the death of GBM cells via the futile MMR repair cycles. MGMT activity has been found in a wide range of both normal and tumour tissues including GBMs (127), and thorough demonstrations have revealed the close association between the expression of MGMT and increased cellular resistance towards TMZ both *in vitro* and *in vivo* in preclinical studies (128). The association between increased MGMT expression and increased resistance towards alkylating agents has long been established (129). MGMT promoters have over 90 CpG dinucleotide sites, and epigenetic silencing of MGMT by CpG methylation of MGMT promoter has been associated with therapeutic sensitivity to TMZ in clinical studies and is a reliable biomarker of sensitivity to TMZ (101, 118). In various studies, treatment with TMZ resulted in prolonged survival in GBM patients when the promoter of MGMT is methylated, and MGMT promoter CpG methylation is observed in approximately 50% of GBM patients (103, 118, 130). The epigenetic silencing of MGMT are also more commonly seen in IDH1/2 mutant tumours, and thus are more common amongst secondary GBM patients (131).

In essence, MGMT is the bane of TMZ chemotherapies, with the expression of MGMT being directly correlated with the chemoresistance of GBM cells. MGMT loses its enzymatic activity after a reaction and undergoes ubiquitination, and thus attempts have been made to aggressively deplete the amount of circulating MGMT like the aforementioned altered schedules to strengthen the effects of TMZ. While the dose-dense schedule allowed for the accommodation of concomitant radiotherapy sessions and increased TMZ exposure, the dose-dense schedule alone did not demonstrate any significant survival advantage beyond just palliative in a phase III trial regardless of the methylation status (132).

MGMT inactivating agents were also attempted in the form of $O^6$-benzylguanine ($O^6$-BG), a notorious novel MGMT inactivating agent used extensively in most experiment trials as well as clinical trials. $O^6$-BG inhibits MGMT by transferring a benzyl group onto the cysteine residue in the active site of MGMT, depleting the protein through subsequent ubiquitination. Preclinical results demonstrated its effectiveness in potentiating the effect of nitrosourea-based alkylating agents such as BCNU in human GBM xenografts (133). In spite of that, the promising preclinical results were not translated in actual clinical trials, with a phase III trial involving $O^6$-BG and TMZ failing to demonstrate any meaningful effect in restoring TMZ sensitivity in GBM patients (134). The concomitant use of $O^6$-BG also came with a significant drawback, as the presence of $O^6$-BG necessitated the need to reduce the dosage of TMZ to account for the severe hematologic toxicities induced by the concomitant use of both agents (134), reinforcing the need to search for alternatives with higher efficiencies.

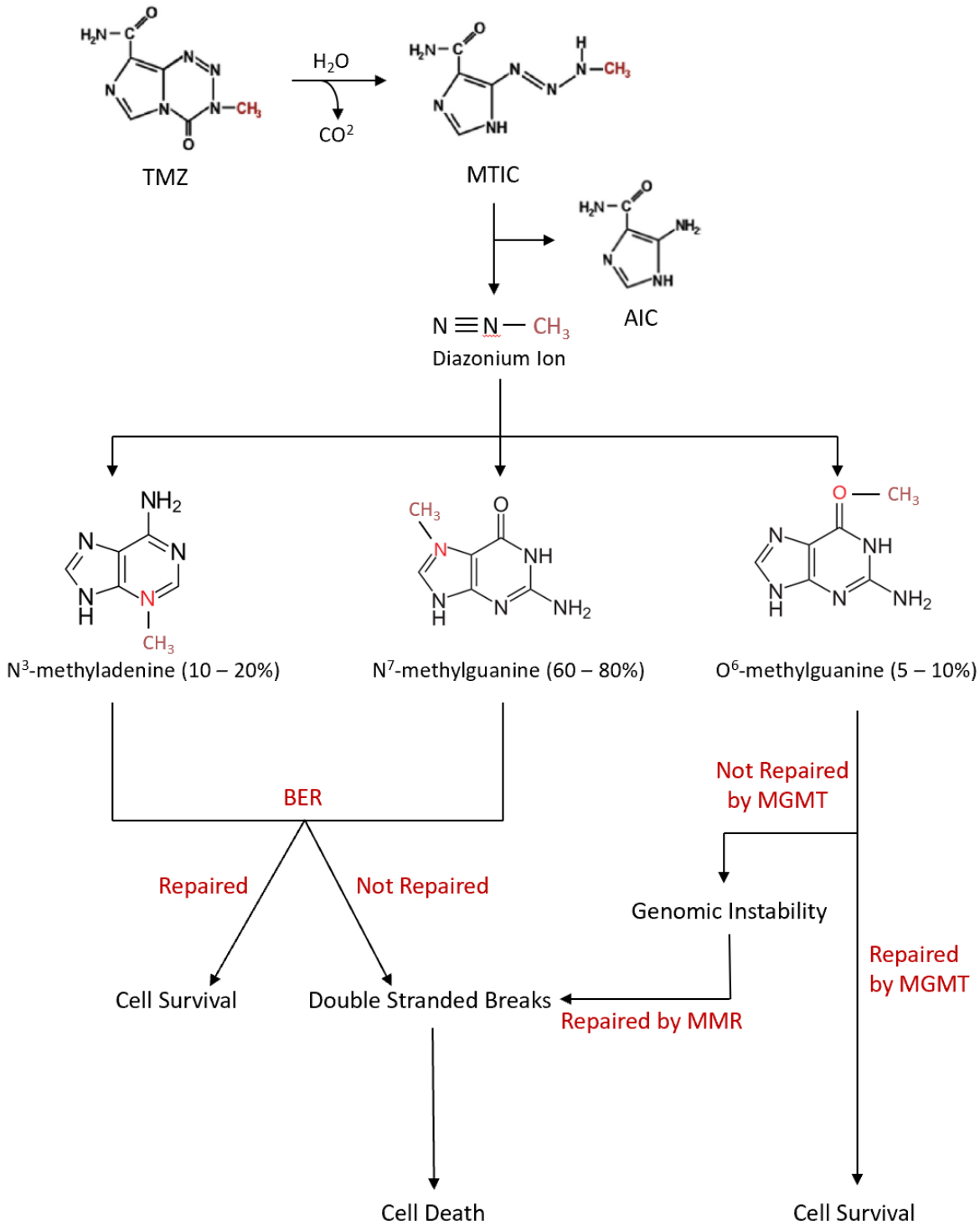



*Figure 2: Temozolomide mode of action and activated DNA repair pathway. TMZ is hydrolysed at physiological pH to form active diazonium ions. The $S_N1$ alkylating reaction by the diazonium ion produced $N^3$-meA, $N^7$-meG and $O^6$-meG. $N^3$-meA and $N^7$-meG are both repaired by the BER system. Any failure in repairing both adducts will lead to double-stranded breaks and subsequently cell death. $O^6$-meG is repaired by MGMT, where the methyl group is removed onto the active site of MGMT in a suicidal reaction that leads to the ubiquitination of the repair protein. Unrepaired $O^6$-meG leads to genomic instability where a futile repetitive repair cycle that eventually leads to double-stranded breaks.*

## 1.4 Potential of Neuropeptides in the Treatment of Cancer

Very little headway has been made to increase the life expectancy of GBM patients which warrants a need to pursue new therapeutic avenues. Therefore, we looked into the potential of neuropeptides as a therapeutic avenue for GBM. One of the most prominent uses of neuropeptide in cancer treatment is somatostatin, also known as growth hormone-inhibiting hormone. Somatostatin is an endogenous cyclic tetradecapeptide hormone that functions as a neurotransmitter, possessing anti-secretory and anti-proliferative effects. Somatostatin peptides are produced from an initially secreted 116 amino acid precursor cleaved to form prosomatostatin, where further endoproteolytic cleavage produces two active forms, somatostatin-28 (SST-28) and somatostatin-14 (SST-14) (135). Both active forms are mostly distributed evenly across the body. However, somatostatin has an extremely short half-life of 1-3 minutes as a result of proteolytic degradation by peptidases in the plasma and tissues (136). Somatostatin, unlike most peptides, has a lack of receptor specificity, able to bind to five different subtypes of its G-protein coupled receptor (SSTR1 – 5) (137). The receptors share 39% - 57% homology in sequence, with the intracellular α-helical regions being highly conserved, differing in the extracellular N-terminal domains (135). Furthermore, binding of somatostatin to the different receptors triggers different reactions, ranging from the inhibition of various hormones to reducing cell proliferation and apoptosis (135). The short half-life and the lack of receptor specificity have thus led to the development of numerous somatostatin analogues.

Somatostatin and its analogues have been used to target tumours such as pituitary adenomas, paragangliomas, carcinoids, breast cancers, malignant lymphoma and small-cell lung cancers (135). Somatostatin acts on tumours directly by inhibiting the growth factor signalling pathway, leading to reduced proliferation, apoptosis induction and inhibition of metastasis (135). This is achieved through the binding of SSTRs that couple to $G_{\alpha i}$, inhibiting the action of adenylate cyclase (135). SSTRs' anti-proliferative effects are a result of direct activation of protein tyrosine phosphatases (PTPs), facilitated by the prior activation of $G_{\alpha i}$ (138). PTPs play a central role in dephosphorylating growth factor bound RTKs (138). Somatostatins are able to block cell cycle progression via G1/S or G2/M cell cycle arrest (138). The effect of somatostatin in a tumour depends on the distribution and activation of the SSTR subtype. Somatostatin is able to reduce proliferation by restoring the functional gap junctions via SSTR2 that are made up of connexins (139). The restoration of the gap junctions allows differentiated cell states to be maintained while inhibiting any cell contact (139). Cx26 and Cx43 are highly influential in the restoration of the gap junction, and both connexins were downregulated in cancer cells (139). Furthermore, the anti-proliferative effect of SSTR2 hinges on the restored synthesis of Cx26 and Cx43

(139). The activation of SSTR2 or SSTR3 induces apoptosis in tumour cells, either via the extrinsic pathway triggered by death receptors or the intrinsic pathway, which involves the mitochondria (140, 141). Activation of SSTR2 in pancreatic cancer cells induced the expression of both TNFR1 and DR4 receptor proteins, which sensitised the cells to TNFα and TRAIL-induced apoptosis (142). It was also shown that SST2 decreased the expression of mitochondrial antiapoptotic protein, Bcl2 (142). Induced apoptosis in breast cancer cells in contrast, was found to be facilitated through the activation of SSTR3, where wild type p53 and pro-apoptotic protein Bax was induced in the absence of the usually present cell cycle arrest (143).

Somatostatin can also exert anti-tumour properties on cancer cells indirectly. Since cancer cells rely on angiogenesis for growth, invasion and metastasis, somatostatin can shut down cancer cells' avenue of growth by inhibiting various angiogenic factors such as VEGF, PDGF and IGF-1 (135). SSTR1 are highly populated in vessels, where it inhibits proliferation, migration, and neovascularisation (138). The generation of new vessels facilitated by the prominent angiogenic factor VEGF can be shut down by the activation of SSTR3 (144). SSTR2 was found to be able to upregulate the expression of thrombospondin-1, which serves as an oncosuppressive agent in pancreatic cancers (145). Somatostatin is also capable of suppressing the proliferation and migration of endothelial cells and monocytes, and in the process impede the production of nitric oxide which are major regulators of the angiogenic process, a critical process that supplies a necessary supply of oxygen and nutrients to facilitate metastasis (146). Furthermore, a study was conducted where it was revealed somatostatin inhibited cell migration in GBM U87 and T98 cell lines by greatly reducing the motility induced by PDGF (147).

Having said that, the function of reproductive neuropeptides in the development of glioma is still largely unanswered. GnRH is a reproductive neuropeptide involved in regulating the secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH). It was found that GnRH and its receptor were found in cancers either related (breast, ovarian and endometrial) or unrelated (urinary bladder, pancreatic cancers and GBM) to the reproductive system (148). Anti-tumour properties including anti-proliferative, anti-metastatic and anti-angiogenesis have been demonstrated in various cancers upon activation of its receptor by GnRH agonists. GnRH antagonists that inhibit the release of LH and FSH in the pituitary intriguingly have the same effect as agonists when administered in a tumour environment, exerting an anti-tumour effect through activation of GnRH-R, subverting the established GnRH agonist/antagonist dichotomy in the pituitary (148, 149). GnRH possesses an altered

structural form in the form of GnRH-II, where its primary structure is largely conserved with three different amino acids (148). The peptide and its putative receptor are found across multiple mammalian species (150, 151). While GnRH-II is indeed present in humans, type-II GnRH-R gene carries a frame shift and a premature stop codon, indicating the absence of a putative functional receptor for GnRH-II in humans (149). It is thus believed that all the functions of GnRH, GnRH-II and their respective analogues are mediated through the activation of type-I GnRH-R. Millar and his group proposed that mutations in the primary structure of GnRH-R affect the receptor's tertiary structure folding, altering the receptor conformational state and changing its ligand affinity and signalling selectivity (152).

The signalling pathway activated by GnRH depends on the cell context. The signalling mechanism in the gonadotropic axis involves coupling with G proteins $G_{\alpha s}$ and $G_{\alpha q}$ which leads to the activation of either protein kinase A (PKA) and adenylate cyclase (AC) or protein kinase C (PKC) and phospholipase C (PLC) respectively upon ligand binding. In contrast, it was found that GnRH inhibits the mitogenic effect of growth factors in cancer cells line by coupling to $G_{\alpha i}$ (153-155). Researchers showed in their studies that reduced proliferation in human endometrial, ovarian and breast cancer cells was induced when administered with GnRH and GnRH-II agonists, while other studies have shown that GnRH and GnRH-II antagonists were found to induce apoptosis in the same cancer cells (148, 149, 156-158). Reduced proliferation by GnRH was achieved through the activation of PTP to counteract the signalling of growth factor receptors (148). Dephosphorylation of EGFRs leads to reduced mitogenic signal transduction which translates to reduced MAPK activation, c-fos expression and EGF-induced proliferation (148). GnRH agonists were also found to operate outside the confines of the mitogenic signal pathway, stimulating the activity of JNK and AP-1 in human endometrial cancer cells, independent of the AP-1 activators, PKC or MAPK/ERK (159). The apoptotic effect induced by GnRH and GnRH-II antagonists were due to a dose-dependent loss of mitochondrial membrane potential and the increased expression of caspase-3 (148). Activating the intrinsic apoptotic pathway, GnRH-II antagonists interacts with the MAPK pathway, activating the expression of stress-induced MAPKs p38 and JNK on a time-dependent basis that ultimately leads to the phosphorylation of Bax (160).

GnRH agonists and analogues were also found to reduce tumour growth and influenced angiogenesis and invasion in GBMs, prostate cancer cells and melanomas (157, 161, 162). Metastasis is a complex process, and GnRH was found to hamper metastasis of prostate cancer cells by modifying actin cytoskeleton organisation, interacting with regulatory proteins involved in actin polymerisation (Rac1,

Cdc42, RhoA) as well as affecting the localisation of αvβ3 integrins, impeding the effects of IGF-1 on cell motility (163, 164). Dissemination of cancer cells require reduced cell adhesion to allow for expansive cell movements, and cell adhesion between cells are maintained by cadherins that mediate $Ca^{2+}$ dependent homotypic cell-cell interactions (165). The introduction of GnRH analogues saw an increase in cadherin expression as well as decreased activities of the plasminogen activator system (involved in the local degradation of the extracellular matrix) in human prostate cancer cells (165, 166). In tumours unrelated to the reproductive system such as melanoma, GnRH agonist goserelin was found to significantly hampered metastasis by reducing the expression of α3-integrin and matrix metalloproteinases-2 (MMP-2) (167). Synthesis and secretion of proangiogenic factors like VEGF were also found to be decreased by the activation of GnRH-R, further hindering the ability of tumour cells to metastasise in melanoma cells (168).

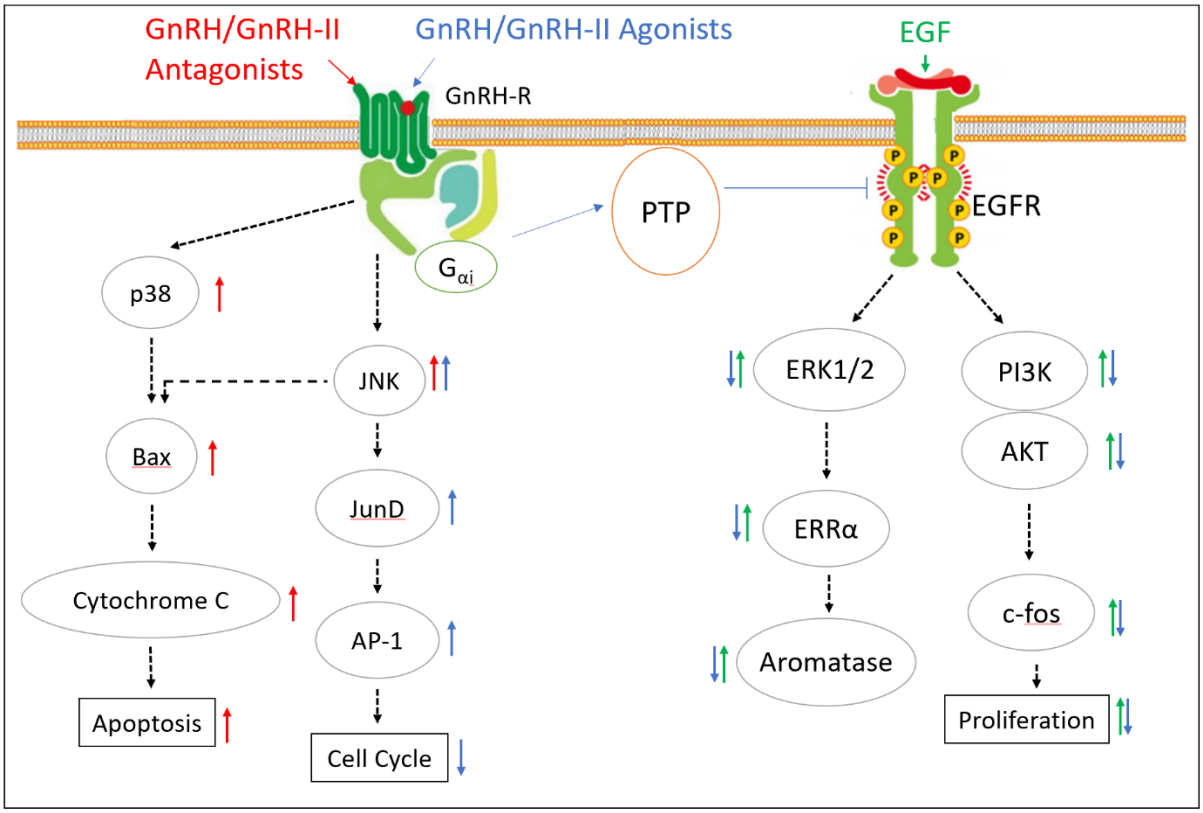



*Figure 3: GnRH's anti-proliferative and apoptotic signal transduction in cancer. GnRH and GnRH-II agonists stimulate the action of PTP, leading to the downregulation of the mitogenic signalling pathway and reduced proliferation. Outside of the confines of the mitogenic signalling pathway, GnRH agonists were also found to stimulate the activity of AP-1 independent of its activators, PKC and ERK. GnRH and GnRH-II antagonists on the other hand, induce apoptosis by increasing the expression of p38 and JNK, which leads to stimulated Bax downstream signalling and increased cell death.*

Kisspeptin, widely known for its anti-metastatic properties, is a neuropeptide that stimulates the release of GnRH with its neurons being in close contact with GnRH neurons (169). Kisspeptin's anti-

metastatic properties were first found when a full-length chromosome 6 (which contains the gene KISS-1 that encodes kisspeptin) was inserted into C8161, a human metastatic melanoma cell line, where metastatic suppression was observed without affecting tumorigenicity (170). Kisspeptin acts on tumours via its specific G-protein coupled receptor (GPCR), GPR54 that triggers the activation of events such as the mobilisation of $Ca^{2+}$ levels and the activation of PKC and p38 MAPK (171). Upon binding with kisspeptin, GPR54 couples to the $G_{\alpha q}$/11 G-protein subfamily, activating key downstream targets such as phospholipase Cβ that generates inositol triphosphate 3 and diacylglycerol as messengers (172).

The specific mechanism in which kisspeptin suppresses metastasis in tumours still eludes our understanding. A group of researchers believed activation of GPR54 lead to the inhibition of calcineurin through the activation of calcineurin inhibitor MCIP-1 (173). Inhibition of calcineurin was found to possess the function of blocking VEGF-induced cell motility and angiogenesis (174). The inhibition of calcineurin in human papillary thyroid cancer cells inhibited its growth and motility *in vitro* (173). It is also believed that the anti-metastatic properties of kisspeptin stem from the inhibition of MMPs. MMP-9 is an essential protease when it comes to promoting tumour metastasis, possessing the ability to degrade primary structures of the extracellular matrix and basement membrane (171). Overexpression of kisspeptin has been found to inhibit NF-κB that interacts with MMP-9 promoter, resulting in diminished MMP-9 expression (175). Furthermore, the presence of Kiss-1 gene also results in the activation of focal adhesion kinase and paxillin, contributing to the formation of focal adhesions and stress fibres that has been found to also contribute to reduced metastasis (176). To date, the ability of kisspeptin to suppress metastasis has been observed in numerous cancers such as melanoma, thyroid, ovarian, bladder, gastric, oesophageal, pancreatic, lung and pituitary cancers (177).

### 1.4.1 Gonadotropin-Inhibitory Hormone (GnIH)/RF-amide Related Peptides (RFRP)

It was widely accepted that the release of gonadotropins was regulated only by GnRH, with no direct influence of other neuropeptides on the reproductive axis (178). This dogma was essentially debunked when GnIH was discovered. GnIH, commonly referred to as RFRP in mammals was first discovered by Tsutsui et al. in the median eminence and paraventricular nucleus (PVN) of a Japanese quail, Coturnix japonica in the early 2000s, inhibiting gonadotropin release by acting directly on the pituitary (179). The bioactive peptide discovered had a sequence as follows: Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-$NH_2$ (SIKPSAYLPLRFa). The final five amino acids form the C-terminal motif LPXRFa to which all peptides orthologous to GnIH adhere to (180).

Human GnIH homologues were first identified in 2009 by Ubuka *et al.* (181). Ubuka *et al.* determined the sequence of RFRPs in humans by first collecting hypothalamic tissues from adult males and females, subjecting the tissues to purification by using an immunoaffinity column conjugated with avian GnIH. The peptides were then subjected to MALDI-TOF mass spectrometry. Two GnIH homologs were identified (Figure 4), with human RFRP-1 having the sequence MPHSFANLPLRFa and human RFRP-3 having the sequence VPNLPQRFa. Multiple sequence alignment between the GnIH precursor protein between quail and human revealed that human RFRP-1 (hRFRP-1) aligned with quail GnIH-RP-1 while human RFRP-3 (hRFRP-3) aligned with a GnIH-like peptide of quail (182). The alignment suggests that human RFRP-3 is the functional homologue of avian GnIH instead of RFRP-1. RFRP immunoreactive neurons were found in the dorsomedial region of the human hypothalamus, which emanated from the infundibulum and terminated in the external layer of the median eminence (181). Furthermore, RFRP immunoreactive axon terminal-like structures were found to be in close propinquity to GnRH neurons in the preoptic area (181).


*Figure 4: Primary structure of the human RFRP precursor polypeptide (GenBank_NM_022150). RFRP precursor polypeptide produces two mature peptides, RFRP-1 and RFRP-3. Both peptides contain a C-terminal LPXRF (X = L or Q) motif. Both peptides are followed by a glycine which provides an amide and acts as a signal for amidation and arginine which facilitates the release of the peptides through endoproteolysis. Figure obtained from Ubuka et al. (181).*

GnIH/RFRP-3 inhibits GnRH neurons in vertebrates, which in turn suppresses the synthesis and release of gonadotropin in the pituitary gland (182). ICV infusion of RFRP-3 in female rats reduced GnRH neuronal activation and GnRH biosynthesis, while similar observations were recorded in male and female mice, regardless of the pubertal status of the female mice (183-185). The reduction in GnRH neuronal activation by RFRP-3 was certified by Ducret *et al.* when they observed the suppression of the firing rate in 41% of GnRH neurons in adult male and female mice *in vitro* (186). They further suggested the direct interaction between RFRP-3 neurons and GnRH neurons in mice when a

reduction in mean firing rate amongst GnRH neurons was observed in the presence of γ-aminobutyric acid receptor antagonists and ionotropic glutamatergic receptor antagonists, which blocks GABA and glutamatergic neurotransmission (186). Their findings allowed the theory of RFRP-3 neurons directly interacting with GnRH neurons to exert its effects without the need for fast GABA or glutamatergic transmissions to ring true.

RFRP-3 neurons are also in close contact with kisspeptin neurons which are responsible for producing kisspeptin that provides an excitatory signal to GnRH neurons (187). Approximately 25% of kisspeptin neurons in the arcuate nucleus expressed GPR147, the receptor for RFRP-3, and approximately 35% of the kisspeptin neurons are in contact with RFRP-3 fibres (188). Using a GnRH neuronal cell model mHypoA-GnRH/GFP, Son *et al*. were able to establish that mouse RFRP-3 inhibits kisspeptin-induced GnRH release in mice (189). Although RFRP-3 was able to inhibit kisspeptin-induced GnRH release in the GnRH neuronal cell model, the effect was not replicated in a GnRH neuronal cell line, GT1-7 (189).

Multiple studies have reaffirmed the status of RFRP-3 as the functional homologue to avian GnIH; thus, its inhibitory role in mammals' reproduction system has been well documented. However, the exact function of RFRP-1 in mammals remains unknown. RFRP-1 has a different C-terminal in the form of LPLRFa, but like RFRP-3 it is expressed both in the hypothalamus and in the gonads (181, 190). RFRP-1 was found to have different potency and effect *in vivo* compared to RFRP-3, despite sharing the same receptor (191-193). Recently, Dave et al. found out that RFRP-1 had a different role in reproduction compared to RFRP-3 when ovaries of a cyclic mouse were treated with RFRP-1 *in vitro*. RFRP-1 was found to stimulate the synthesis of estradiol, whereas the inhibitory effect of RFRP-3 on gonadal steroidogenesis is well documented (190). To this day, the function of RFRP-1 remains a conundrum.

Like its counterparts in the HPG axis, RFRP-3 acts directly on gonadotropes via its GPCR which is mainly GPR147. A blastp run showed that GPR147 is very well conserved across vertebrates, with human GPR147 sporting an 87% or higher homology across most vertebrates. GPCRs for RFRP-3 were first identified by Hinuma et al. where they found a cDNA that encoded a GPCR which responded to RFRP-1 and RFRP-3. The seven-transmembrane receptor was named OTGT022, which corresponds to GPR147 (194). Using *in situ* hybridisation and immunocytochemistry, Ubuka *et al.* were able to pinpoint the expression of cognate GPR147 receptors in the human hypothalamus as well as the pituitary (181).

Son et al. attempted to determine the RFRP-3 cell signalling using a mouse gonadotrope model in the form of LβT2 cell line that exhibits all the characteristics of a fully differentiated gonadotrope. FSK and GnRH-induced CRE-luciferase activity were significantly reduced by MDL (AC inhibitor), and consistent with previous findings, mouse RFRP-3 inhibited GnRH-induced increase in CRE-luciferase activity, demonstrating the direct inhibition of GnRH-induced cAMP production by RFRP-3 (195). Son et al. established that PKC inhibitor GF did not influence GnRH-stimulated ERK phosphorylation, while MDL and H89 (PKA inhibitor) inhibited GnRH-stimulated ERK phosphorylation to the basal level in LβT2 cells. RFRP was also shown to inhibit GnRH-stimulated ERK phosphorylation as well as the inhibition of GnRH- stimulated intracellular free $Ca^{2+}$ generation (195, 196). Son and his colleagues were able to replicate similar observations in a mouse GnRH neuronal cell line as well (189). These experiments proved that RFRP-3 specifically inhibits GnRH via the AC/cAMP/PKA pathway by coupling to $G_{\alpha i}$, preventing the activation of ERK1/2 signalling and the mobilisation of intracellular $Ca^{2+}$, both indispensable components in the expression of LHβ (Figure 5).

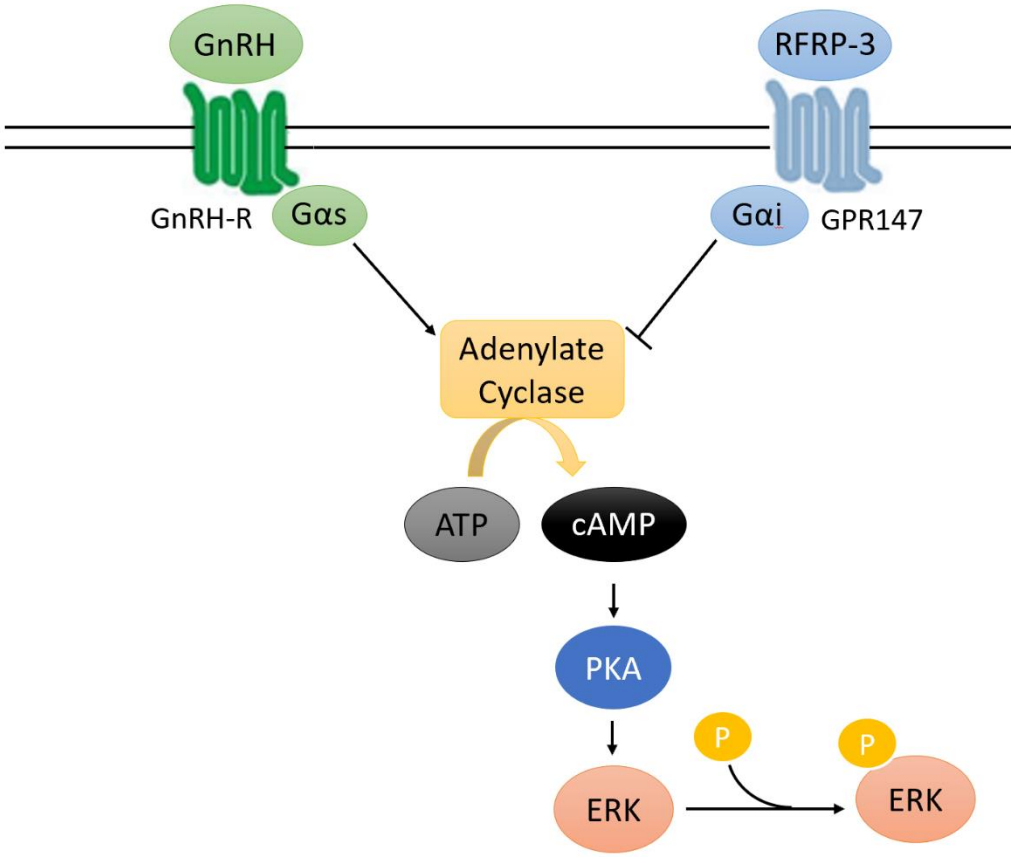



*Figure 5: A model of the signalling pathway activated by GnIH/RFRP-3 upon binding to GPR147. RFRP-3 inhibits GnRH via the AC/cAMP/PKA pathway, which results in a decrease in the phosphorylation of ERK in the MAPK pathway. Phosphorylated ERKs are responsible for the transcription of LHβ, FSHβ as well as the common α genes in the gonadotropes.*

RFRP-3 is an everlasting presence in the regulation of GnRH release. Despite the fact that RFRP-3 neurons being in close proximity to GnRH neurons with the RFRP-3 neuronal axon terminals contacting GnRH neurons that express RFRP-3's receptor, very little is known about its function in a different cell context, especially in cancer cells (187, 189). RFRP-3 is not only an essential cog in the signalling complex occupied by GnRH and kisspeptin, but like its counterparts, RFRP-3 exerts its functions via its specific GPCR. The precedent set by GnRH and kisspeptin which has proven to reduce proliferation and metastasis in different cancers and tumours indicates that RFRP-3 might be a potential therapeutic avenue that was left unexplored in cancers as well. Feve *et al.* revealed in their comprehensive GPCR analysis of GSC and GBM cells that GPR147 was indeed expressed in GBM cells (197). This opens up the possibility of RFRP-3 displaying anti-tumour properties directly through its specific GPCR. Determining the effect of RFRP-3 in GBM can potentially lead to a better understanding of RFRP-3's role in GBM, which might open avenues to developments of another course of action in the fight against GBM.

## 2. Research Aims

The role of RFRP-3 in cancer cells is largely unexplored. RFRP-3 exists within the same signalling complex as GnRH and kisspeptin, playing an essential role in regulating the synthesis and release of GnRH, where GnRH's role as an anti-cancer agent is pretty well established. In cancer cells, GnRH and kisspeptin exhibits anti-proliferative, apoptotic, and anti-metastatic effects via their respective GPCRs. Like GnRH and kisspeptin, RFRP-3 binds to and activates its specific GPCR, GPR147. We hypothesize that RFRP-3 may be a viable therapeutic avenue for GBM, exerting any potential effects via GPR147. Our research project therefore aims to determine any effect that RFRP-3 may have on GBM cells. Therefore, several functional studies were proposed for the completion of our aim:

**(a) To establish the presence of GPR147 in GBM cell lines:**

Using PCRs and Q-PCRs to identify the presence of GPR147 in different GBM cell lines at the transcriptomic level. This is followed up by Western Blot analyses to determine the presence fully translated GPR147s at the proteomic level.

**(b) To determine the active status of GPR147 in GBM cells:**

This was done using cAMP assays to specifically observe any decrease in cAMP concentration that may point to the activation of $G_{\alpha i}$ by active GPR147 receptors upon binding of RFRP-3.

**(c) Identify any possible anti-proliferative or apoptotic effects by RFRP-3 on GBM cells**

To achieve this aim, MTT assays were used to observe any changes to the viability of GBM cells upon administration of RFRP-3. Furthermore, the application of flow cytometry allows for a quantitative look at any possible RFRP-3's influence on the viability and proliferation of GBM cells.

**(d) Observe the viability of RFRP-3 in potentiating the effects of TMZ**

Looking into the expression of the bane of TMZ treatments, MGMT will give valuable insights to the viability of RFRP-3 in enhancing the efficacy of TMZ in GBM cells. Concomitant treatments of RFRP-3 and TMZ will reveal the feasibility of applying RFRP-3 in future GBM treatments.

# 3. Materials and Methods

## 3.1 Cell Culture

Primary glioblastoma cells LN18 (ATCC® CRL-2610) were maintained in RPMI 1640 with L-Gln media (Nacalai Tesque Inc., Kyoto, Japan) with 10% FBS (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (MilliPoreSigma, Burlington, MA, USA) in T25 culture flasks (NEST Biotechnology Co., Wuxi, China). Primary glioblastoma cells A172 (ATCC® CRL-1620) and T98 (ATCC® CRL-1690) cells were cultured in DMEM culture media (MilliPoreSigma) with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin (MilliPoreSigma). Cells were grown in an incubator at 37°C with a humidified atmosphere of 5% $CO_2$. All cells were maintained between passage 10 to 30.

## 3.2 Determination of GPR147 & MGMT expression in GBM Cell Lines

The expression of GPR147 and MGMT at the transcriptomic level were determined using two sets of primers, respectively (refer to Table 5 for the sequence). GPR147 and MGMT expression levels were determined in 3 different GBM cell lines, namely LN18, T98 and A172. GPR147 and MGMT were amplified with polymerase chain reaction (PCR) (refer to Table 6 for the PCR reaction) using 5x i-PCR Red Master Mix (iDNA Biotechnology Co., KL, Malaysia) and the PCR products were imaged under UV ray after being subjected to electrophoresis in 2% agarose gel.

Table 5: Sequence of the primers used for the determination of GPR147 (NM_022146) and hMGMT (NM_002412) in the three different GBM cell line as well as GADPH (AF261085) used as the internal housekeeping control.

| | *Primers* | *Sequence* | *Product Length (bp)* |
|---|---|---|---|
| *hGPR147* | Forward | TTGGCCCCTAAGTCAGAATG | 290 |
| | Reverse | ACCCAGTGATGAGGTTGTCC | |
| *hMGMT* | Forward | AGCAAGAGTCGTTCACCAGACA | 90 |
| | Reverse | GGGCTGCTAATTGCTGGTAAGA | |
| *hGADPH* | Forward | GGAAGGTGAAGGTCGGAGTCA | 101 |
| | Reverse | GTCATTGATGGCAACAATATCCACT | |

*Table 6: Details of the PCR cycle used in the amplification of GPR147 and hMGMT gene in the three different GBM cell lines.*

| | TEMPERATURE | TIME |
|---|---|---|
| **HOLD** | 95°C | 2 minutes |
| **CYCLE** | 95°C | 30 seconds |
| **(35 FOR GPR147)** | 55°C | 30seconds |
| **(30 FOR HMGMT)** | 72°C | 1 minute |
| **EXTENSION** | 72°C | 2 minutes |

### 3.3 RNA Isolation and cDNA Synthesis

cDNA of the three GBM cell lines were obtained by first dissolving the cells in 1ml of TriZol reagents. Chloroform was added in a 1:5 ratio (Chloroform: Trizol) and is mixed thoroughly, followed by centrifugation at 12000G, 4°C for 15 minutes. The aqueous phases were carefully removed into fresh Eppendorf tubes, and isopropyl alcohol was added at a 1:2 ratio (Isopropyl alcohol: Aqueous phase). The mixtures were incubated at room temperature for 10 minutes followed by centrifugation at 12000G, 4°C for 15 minutes. Supernatants were discarded and any pellets produced were rinsed with 75% ethanol, followed by centrifugation at 7500g, 4°C for 5 minutes with the rinsing step being repeated twice. The pellets were then left to air dry for 5 minutes to remove any residue ethanol, and the pellets were then subsequently dissolved in 25ul of MilliQ water. The purity of the RNAs extracted was determined using ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA isolations that had a 260/280 value of above 1.80 were deemed pure. 500 to 1000ng of RNAs were then converted into cDNAs using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forster, CA, USA). 500ng was used for the MGMT gene expression tests while 1000ng of RNAs were used for the quantification of GPR147 in GBM cells.

### 3.4 Quantification of GPR147 and MGMT mRNA using Q-PCR

The DNA fragment copy number of GPR147 and MGMT genes was determined using the primers listed in Table 5. PCR products produced by GPR147 and MGMT primers were ligated into pGEM-T Easy Vectors (Promega, Madison, WI, USA). The ligated plasmids were then transformed into competent DH5α *Escherichia coli* cells to amplify the number of plasmids. The plasmids were subsequently harvested from the *E. coli* cells using Wizard SV Minipreps DNA Purification System (Promega). The cloned vectors were then subjected to Sanger sequencing to confirm the sequence of the cloned GPR147 and MGMT fragment using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and 3310 Genetic Analyzer (Applied Biosystems).

SensiFAST SYBR Hi-ROX Kit (Bioline, Taunton, MA, USA) was used to perform quantitative PCR with the PCR cycle as follow: 95°C for 2 minutes, 95°C for 15 seconds and 60°C for 30 seconds repeated for 40 cycles and a final dissociation for melting curve analysis. A standard curve for both genes was generated by preparing dilutions of both plasmids at a dilution factor of 10 for eight times, and the dilutions were subjected to quantitative-PCR. The copy number of the GPR147 and MGMT mRNAs was determined according to the generated standard curves with the primer efficiency coming in at 88.4%.

### 3.5 Western Blot Analysis

Cultured LN18, A172 and T98 cells were washed with ice-cold PBS before being the addition of 100µl of lysis buffer along with 1ul of protease inhibitor. The mixture is transferred into an Eppendorf tube and subjected to centrifugation at 16000rpm, 4°C for 20 minutes. The supernatant was retained in a fresh tube and kept at -20°C until further use. Bradford assay was performed to determine the total concentration of protein in the cell lysates. The standard curve was determined by preparing eight dilutions of bovine serum albumin (BSA), and the absorbance was recorded using TECAN Infinite 200 Pro microplate reader. The concentration of protein in the cell lysates were then determined according to the obtained standard curve.

SDS-PAGE gels were prepared using a 12% resolving gel and a 4% acrylamide stacking gel. 40mg of protein were loaded into each well of the SDS-PAGE gel. The proteins were separated at 200V for 20 minutes before the separated proteins were blotted onto a nitrocellulose membrane via a semi-dry transfer. The membrane was then blocked using 2% skimmed milk for an hour before being incubated using the primary antibody (GPR147, ab140906, 1:1000 dilution, ABCAM, Cambridge, United Kingdom) in 0.5% BSA overnight followed by secondary antibody (1:1000 dilution) for 2 hours. The membrane was then imaged using a CCD detector.

### 3.6 cAMP Assay

$5x10^3$ cells/well were seeded into white, clear-bottomed 96 well plates and were incubated overnight. The cells were then starved overnight in serum-free media to reduce the basal cAMP expression. Treatment of cells with 1µM, 10µM and 100µM of RFRP-3 (VPNLPQRF, 1st BASE, Malaysia) in RPMI 1640 with L-Gln media with 0.5% FBS and 1% penicillin/streptomycin in the presence of 3-isobutyl-1-methylxanthine (IBMX, Sigma Aldrich) for 4 hours. The assay is then performed according to the

manufacturer's protocol to obtain the change in cAMP expression (cAMP-Glo Assay, Promega). Fluorescent reading was then obtained via TECAN Infinite 200 Pro microplate reader.

### 3.7 Cell Viability Assay (MTT Assay)

$5x10^3$ LN18 cells/well were seeded into 96 well plates (NEST Biotechnology Co., Wuxi, China) for MTT assays and were incubated for 24 hours to allow the cells to grow and adhere to the surface of the wells. The cells were starved for 4 hours in serum-free media prior to incubation with RFRP. In the RFRP treatment studies, cells were incubated with RFRP-1 (MPHSFANLPLRF, 1st BASE, Seri Kembangan, Selangor, Malaysia) and RFRP-3 in RPMI 1640 with L-Gln media with 0.5% FBS and 1% penicillin/streptomycin at varying concentrations (1nM, 10nM, 10nM, 1µM, 10µM, and 100µM) up to 24 or 48 hours. Cells were washed with PBS upon changing of media. MTT with initial concentrations of 5mg/ml diluted tenfold in plain RPMI media then replaces the treatment media at the end of the treatment. The cells were incubated for 4 hours to allow the production of formazan before the crystals were dissolved in dimethyl sulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA). The fluorescence of the treated cells was then measured at 570nm using TECAN Infinite 200 Pro microplate reader.

### 3.8 Flow Cytometry

Prior to treatment, $2x10^6$ cells/dish in RPMI 1640 with L-Gln media with 5% FBS and 1% penicillin/streptomycin were seeded in 60mm culture dishes. The cells were incubated overnight and subsequently starved in serum-free media for four hours, followed by treatment with RFRP-3 (1µM, 10µM and 100µM) in RPMI 1640 with L-Gln media with 0.5% FBS and 1% penicillin/streptomycin for 48 hours. After treatment, the cells were harvested via the addition of accutase (Nacalai Tesque) followed by the addition of 1ml of RPMI 1640 complete media after the dissociation of the cells. The cells were then transferred into 15ml centrifuge tubes and centrifuged at 232G, 4°C for 5 minutes. The supernatants were discarded, and the pellets were resuspended in 1x D-PBS. Flow cytometry was then performed using Muse Count & Viability Kit (Luminex, Austin, TX, USA) and Muse Ki67 Proliferation Kit (Luminex) according to the manufacturer's protocol.

### 3.9 Concomitant Treatment of TMZ & RFRP-3

For the identification of TMZ $IC_{50}$ in LN18, the cells were subjected to various concentrations of TMZ (125µM, 250µM, 375µM, 500µM, 625µM, 750µM, and 875µM) for 48 hours. Tumour cells were

divided into two groups (TMZ alone, TMZ and RFRP-3 combined) and treated for 48 hours in the concomitant treatment. The concentration of TMZ used consists of the $IC_{50}$ value and two additional value that is half of its previous value while the concentration of RFRP-3 ranged from 1nM to 100µM. Cells were washed with PBS upon changing of media. MTT assay was then performed at the end of the treatment.

### 3.10 MGMT Gene Expression in The Presence of TMZ

$6x10^5$ cells/well were seeded into 12 well plates (NEST Biotechnology Co.) for MGMT gene expression studies and were left to incubate for 24 hours. The cells were then starved for 4 hours and were incubated with RFRP-3 in RPMI 1640 with L-Gln media with 0.5% FBS and 1% penicillin/streptomycin at varying concentrations (100nM, 1µM and 10µM) up to 48 hours for the RFRP studies. TMZ treatment studies involved three treatment arms (RFRP-3 only, TMZ only, RFRP-3 and TMZ combined) at the TMZ $IC_{50}$ concentration. After the incubation period, the cells were washed with PBS and TriZol reagent was added into each well. The RNAs were then isolated, and 500ng of RNA was converted into cDNA. The relative expression of MGMT mRNA was determined using quantitative PCR with GADPH gene as the internal control using the double ΔCt method.

### 3.11 Statistical Analysis

Statistical analysis was done using IBM SPSS Statistics v24 (IBM, NY, USA). Independent sample t-test and one-way ANOVAs were performed between the control group and the treated groups depending on the treatment. The data were represented as the mean ± standard error mean (SEM). Any p-value less than 0.05 was deemed statistically significant.

# 4. Results

## 4.1 Expression of GPR147 in Three Different GBM Cell Lines

With GPR147 being the active receptor for RFRP-3, identifying the presence of the receptor is paramount before any proceeding work. Expression of GPR147 was examined in three different cell lines, namely LN18, T98 and A172 at the transcriptomic level using regular PCR. Figure 6 shows that GPR147 mRNA is expressed in all three cell lines, with LN18 and T98 cell lines (both resistant to TMZ) both having more intense bands than A172 cell line (sensitive to TMZ). Q-PCR was then used to provide quantitative and concrete insights into the expression of GPR147 in the three cell lines. A standard curve was then used to determine the copy number of GPR147 mRNA in the three different GBM cell lines. It was found that LN18 cells and T98 cells had a slightly higher copy number of GPR147 mRNA (78 and 56 respectively) when compared to A172 cells (20) (Figure 7).

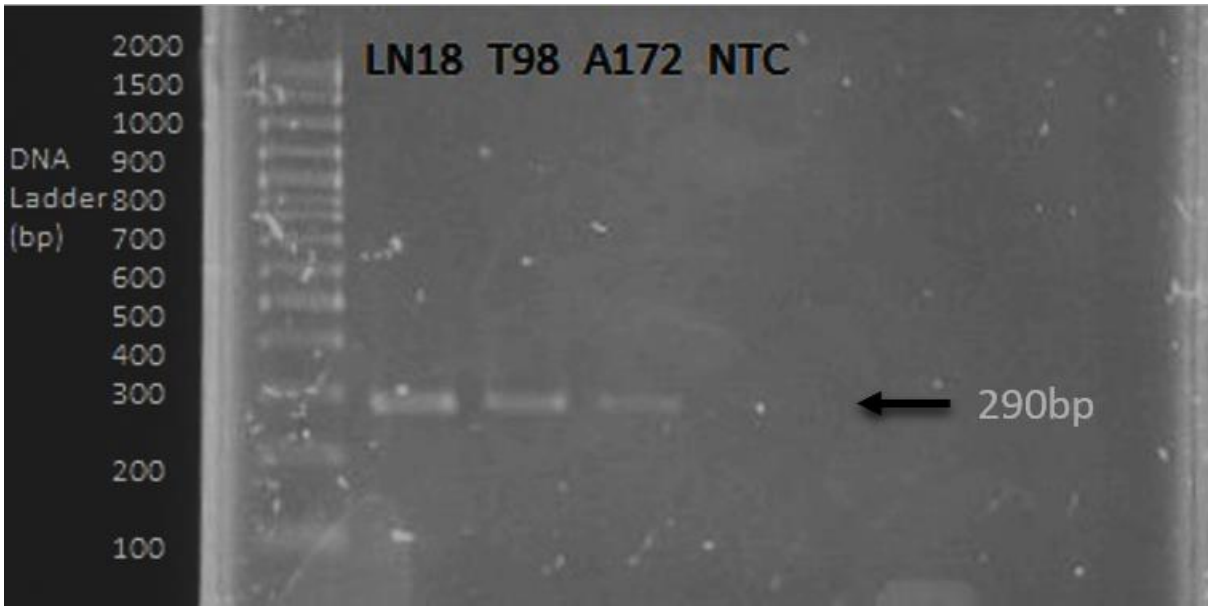



*Figure 6: Gel image of GPR147 mRNA produced by PCR in three different GBM Cell Line. LN18 and T98 are both resistant to TMZ, while A172 cells have a higher susceptibility towards TMZ. The primers used for this PCR produces amplicons at a length of 290bp, which are indicated by an arrow.*

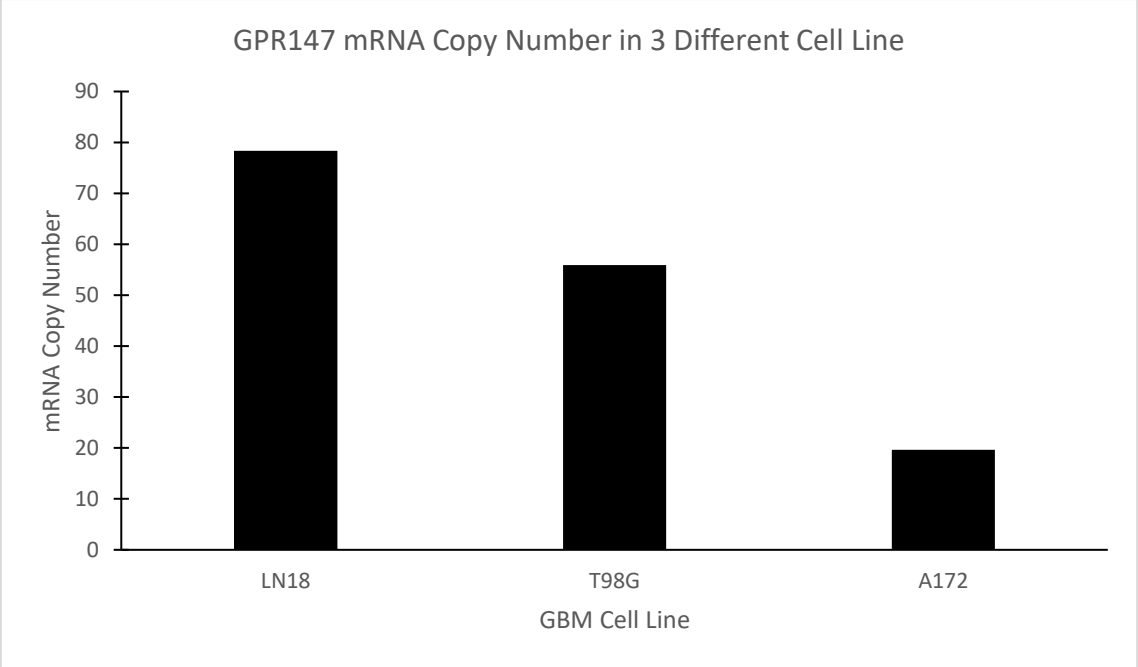



*Figure 7: Graph of GPR147 mRNA copy number in LN18, T98 and A172 cells. LN18 and T98 cells are resistant to TMZ while A172 cells are sensitive to TMZ*

It was established that GPR147 is expressed at the transcriptomic level. To determine the expression of GPR147 at the proteomic level, Western Blot was performed where specific antibodies were conjugated to the protein of interest and imaged under a CCD detector. The expected size of GPR147 comes in at 48kDa. Figure 8 showed that the presence of GPR147 receptors in LN18 and T98 cells with 48kDa bands visible for both cell lines. A172 cells on the contrary saw no translation of the mRNA into GPR147 receptors despite the presence of mRNAs at the transcriptomic level.

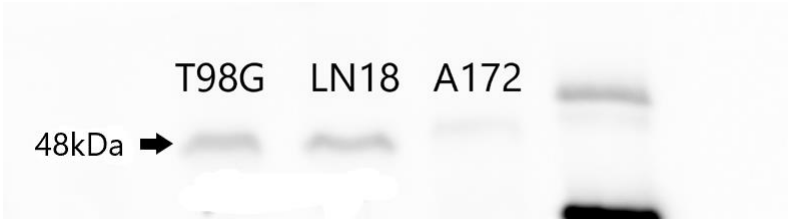


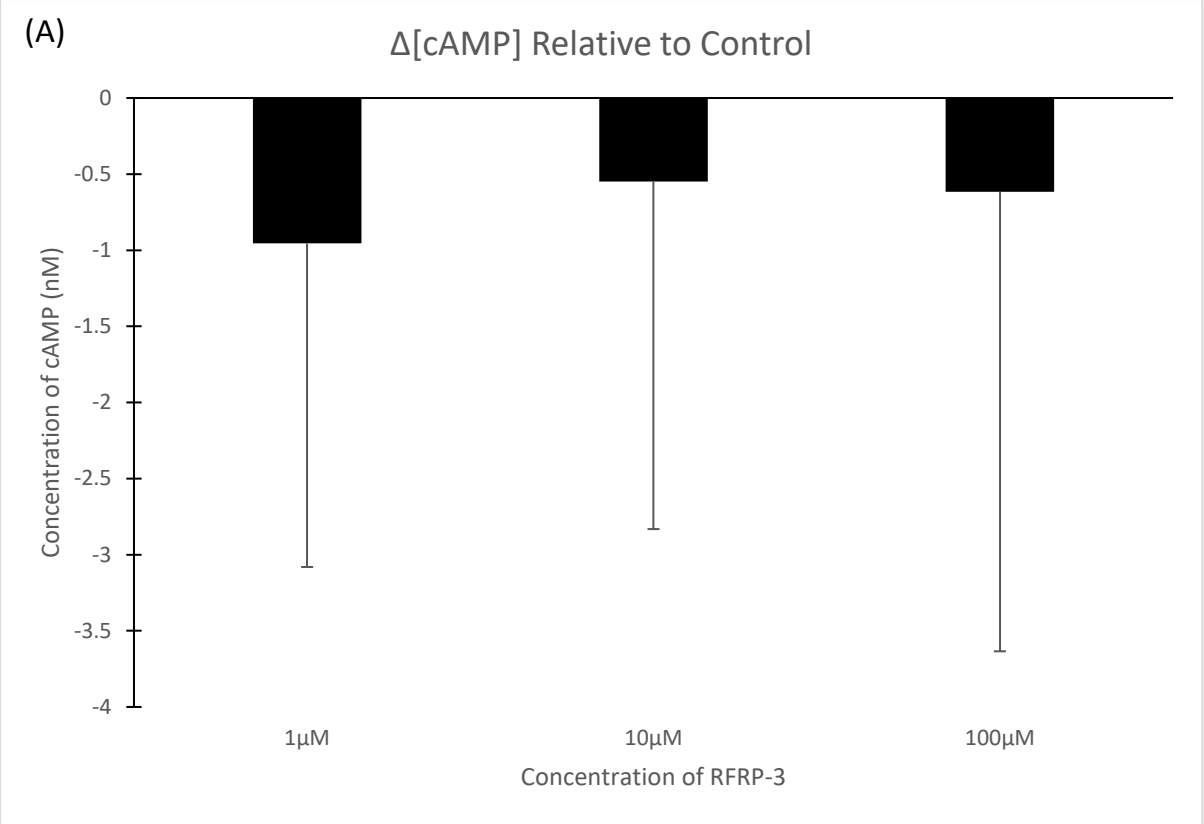

*Figure 8: Results of the Western Blot for GPR147 in T98, LN18 and A172 cells. Black arrow indicates the presence of GPR147 at 48 kDa. GPR147 receptors are expressed in both T98 and LN18 cells but not in A172 cells.*

### 4.2 cAMP Assay

We established the presence of GPR147 in LN18 and T98G cells, but whether the GPCRs expressed were in an active state remains a question. Thus, we ran a series of cAMP assays to find an answer to that question since GPR147 inhibits cAMP accumulation by binding to Gαi. Since LN18 had the highest expression of GPR147, we decided to focus on LN18 cells moving forward. The cells were incubated for 4 hours with 1µM, 10µM and 100µM of RFRP-3 before any relative changes in cAMP concentrations compared to the control was determined.

Using A172 cells as a negative control, we showed that treatment with RFRP-3 had little effect on the concentrations of cAMP. A172 cells treated with RFRP-3 reduced the cAMP concentration with no signs of a dose-dependent reduction in cAMP concentration. (Figure 9A). When treated with 1µM, 10µM and 100µM of RFRP-3, cAMP was decreased by 0.95nM, 0.54 and 0.61nM respectively when compared to the control. The lack of statistically significant data reaffirmed the lack of RFRP-3 activity in A172 cells due to the lack of GPR147 receptors.

Treatment of RFRP-3 in LN18 cells in contrast, saw a higher decrease in the cAMP concentration (Figure 9B). The concentration of cAMP was decreased by 1nM, 1.64nM and 2.8nM when compared to the control respectively after being treated with 1µM, 10µM and 100µM of RFRP-3. A discernible trend was also displayed in the form of a dose-dependent decrease in cAMP concentration when treated with RFRP-3. Unfortunately, while the result does show a dose-dependent decrease in cAMP concentration, the results were not significant statistically.



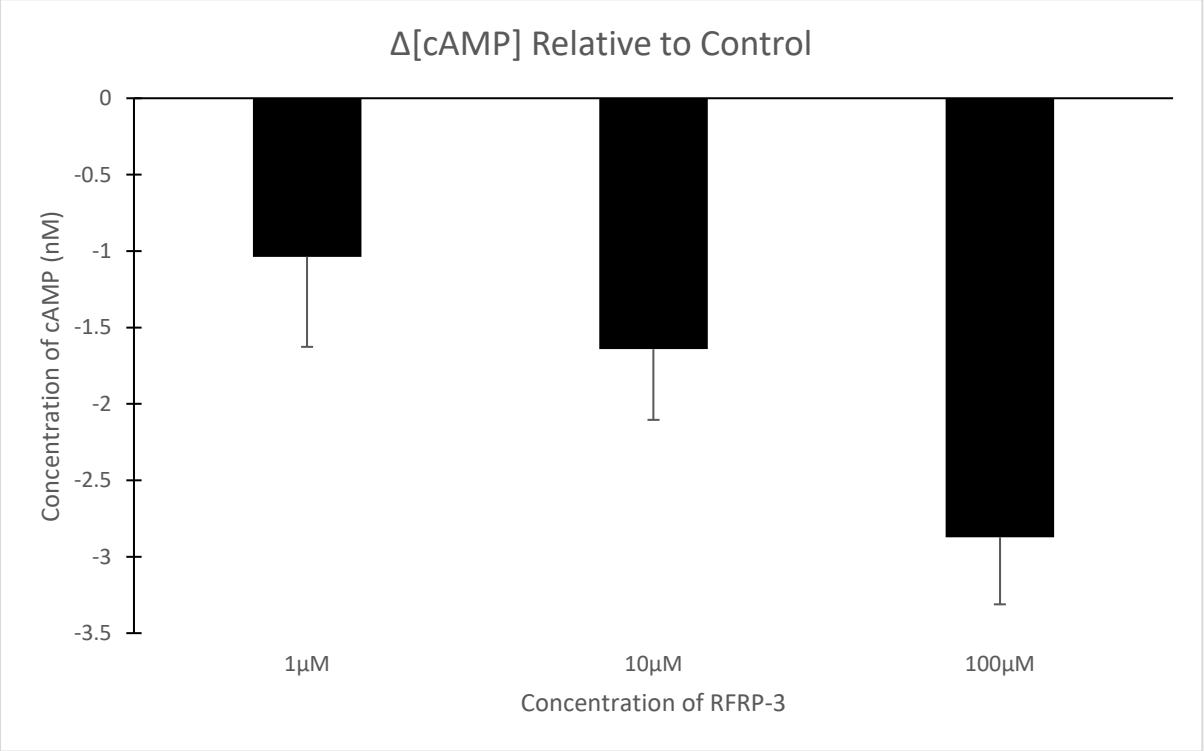
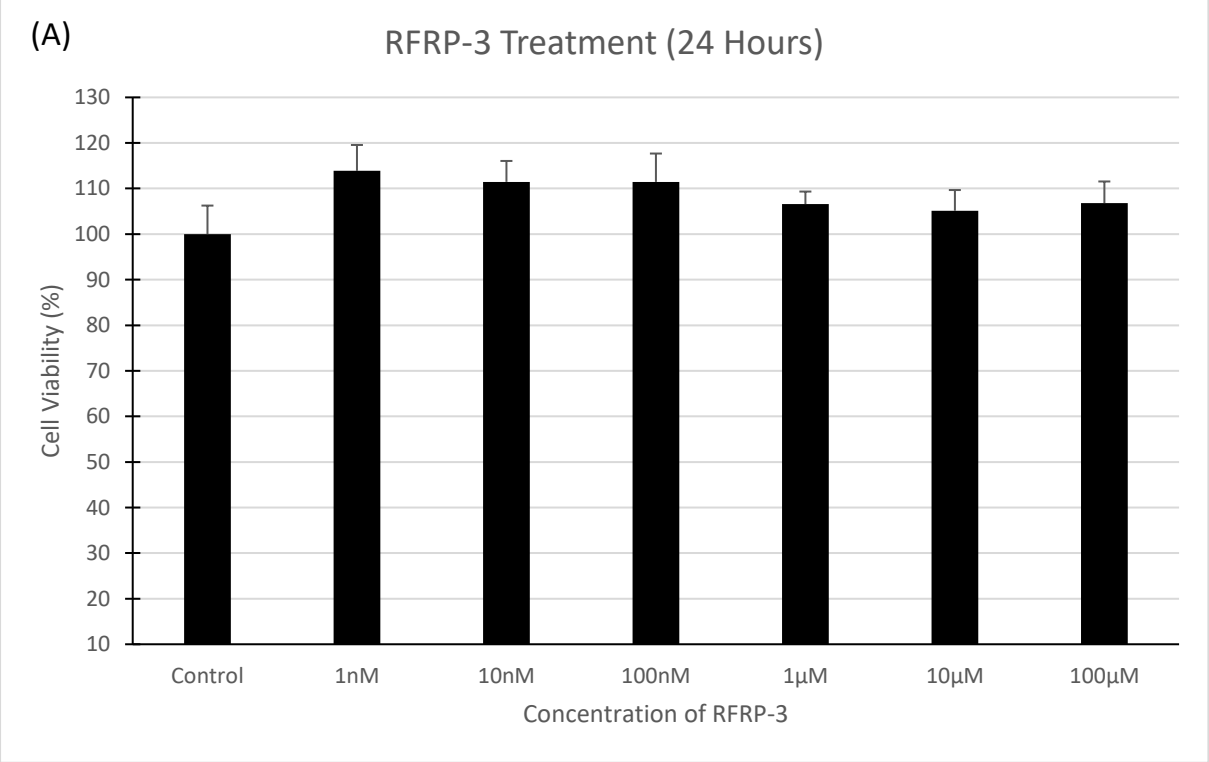




*Figure 9: cAMP Assay measuring the change in cAMP concentration in (A) A172 cells and (B) LN18 cells relative to their respective control after being treated with RFRP-3. Control is the basal expression of cAMP in the cells without the influence of RFRP-3. All changes in cAMP concentration were measured according to the change in luminescence in the treatments relative to their respective control. All data are shown as the mean ± SEM from triplicated experiments. Independent samples t-tests were performed between the treated cells and their respective control groups. No statistically significant result was produced, although it has to be pointed out that 100μM of RFRP-3 did produce a p-value of 0.076 in LN18 cells.*

### 4.3 Cell Viability Assay

Upon the validation of GPR147 expression in GBM cells, we proceeded to determine the effect of human RFRP peptides (RFRP-3 and RFRP-1) on the viability of the cells upon administration in LN18 cells. LN18 cells were subjected to both RFRP peptides treatments at various concentrations for a duration of 24 and 48 hours.

Initial treatment of LN18 cells with RFRP-3 for 24 hours across various concentrations (1nM, 10nM, 100nM, 1µM, 10µM and 100µM) saw no changes in cell viability when compared to the controls (Figure 10A). Treatment of LN18 cells with RFRP-3 for 24 hours recorded increases in viability by 13.9%, 11.5%, 11.5%, 6.6%, 5.1%, and 6.8% respectively. However, no statistically significant results were produced. We then sought to determine the effect of RFRP-3 on LN18 cells after 48 hours. When the treatment was increased by a further 24 hours, our results once again recorded a lack in any significant changes in viability across all the treatments. (Figure 10b). The viability of the cells was 98.9%, 107%, 105.9%, 103.8%, 106.1% and 100.8% respectively upon exposure to RFRP-3 for 48 hours. Administration of RFRP-3 peptides produced statistically insignificant changes in the viability of RFRP-3, and this led us to believe that RFRP-3 had no effect on the viability of LN18 cells.



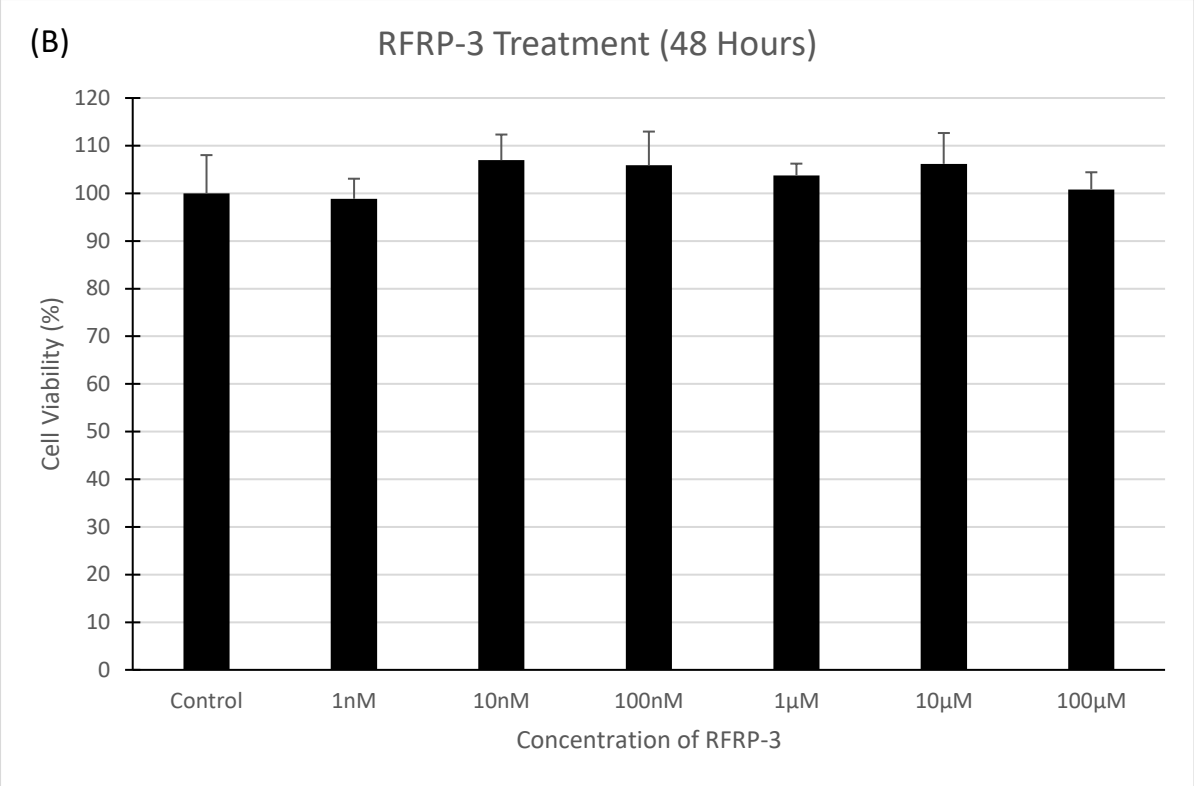



*Figure 10: (A) Cell viability of LN18 cells treated with RFRP-3 for 24 hours. Treatment with any concentration of RFRP-3 showed no statistically significant result. (B) Cell viability of LN18 cells treated with RFRP-3 for 48 hours. No statistically significant result was produced. All data are shown as the mean ± SEM from triplicated experiments. Independent samples t-tests were performed between the treated cells and their respective control groups.*

Although RFRP-3 is the functional homolog of GnIH peptides in birds, the RFRP precursor protein in humans produces another active peptide in the form of RFRP-1 (181); thus we sought to determine the effect of RFRP-1 on LN18 as well. To determine the effect of RFRP-1 on LN18 cells, treatment was done on LN18 cells at various concentrations ranging from 1nM to 100µM for 24 and 48 hours. Initial 24 hours treatment revealed RFRP-1 had little effect on the viability of LN18 cells except cells treated with 100µM of RRP-1 (Figure 11A). Viability of LN18 cells after RFRP-1 treatment were 98.7%, 93.6%, 99.7%, 100.6%, 95.4% and 86.2% respectively. LN18 cells treated with 100µM of RFRP-1 saw the highest decrease in viability; however, the result was statistically insignificant.

We then sought to determine the effect of RFRP-1 on LN18 cells after 48 hours. A similar trend was observed where RFRP-1 had little effect on the viability of LN18 cells (Figure 11B). The viability recorded was as follows: 98.7%, 94.8%, 104.9%, 102.5%, 105.3%, 80.2% respectively. Similar to the 24 hours treatment, cells treated with 100µM of RFRP-1 saw a 20% decrease in viability. However, like the 24 hours treatment, the result was also statistically insignificant. Overall, this suggested that

neither RFRP-3 nor RFRP-1 had any effect on the viability of LN18 cells, suggesting the lack of involvement in inducing any cytotoxic effect in LN18 cells.

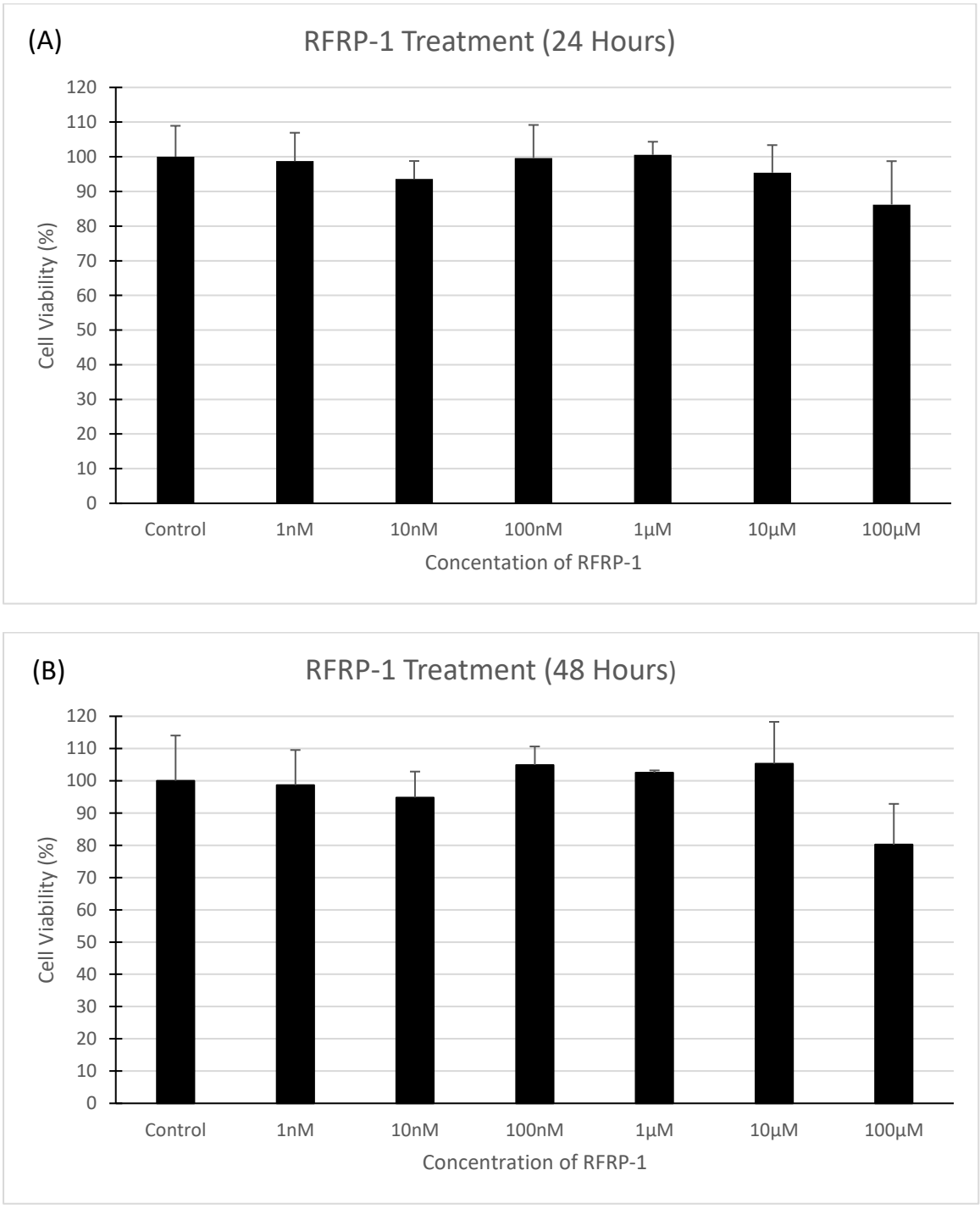



*Figure 11: (A) Cell viability of LN18 cells compared to the control after being treated with RFRP-1 for 24 hours. (B) Cell viability of LN18 cells compared to control after being treated with RFRP-1 for 48 hours. No statistically significant results were produced. All data are shown as the mean ± SEM from triplicated experiments. Independent samples t-tests were performed between the treated cells and their respective control groups.*

### 4.4 Flow Cytometry

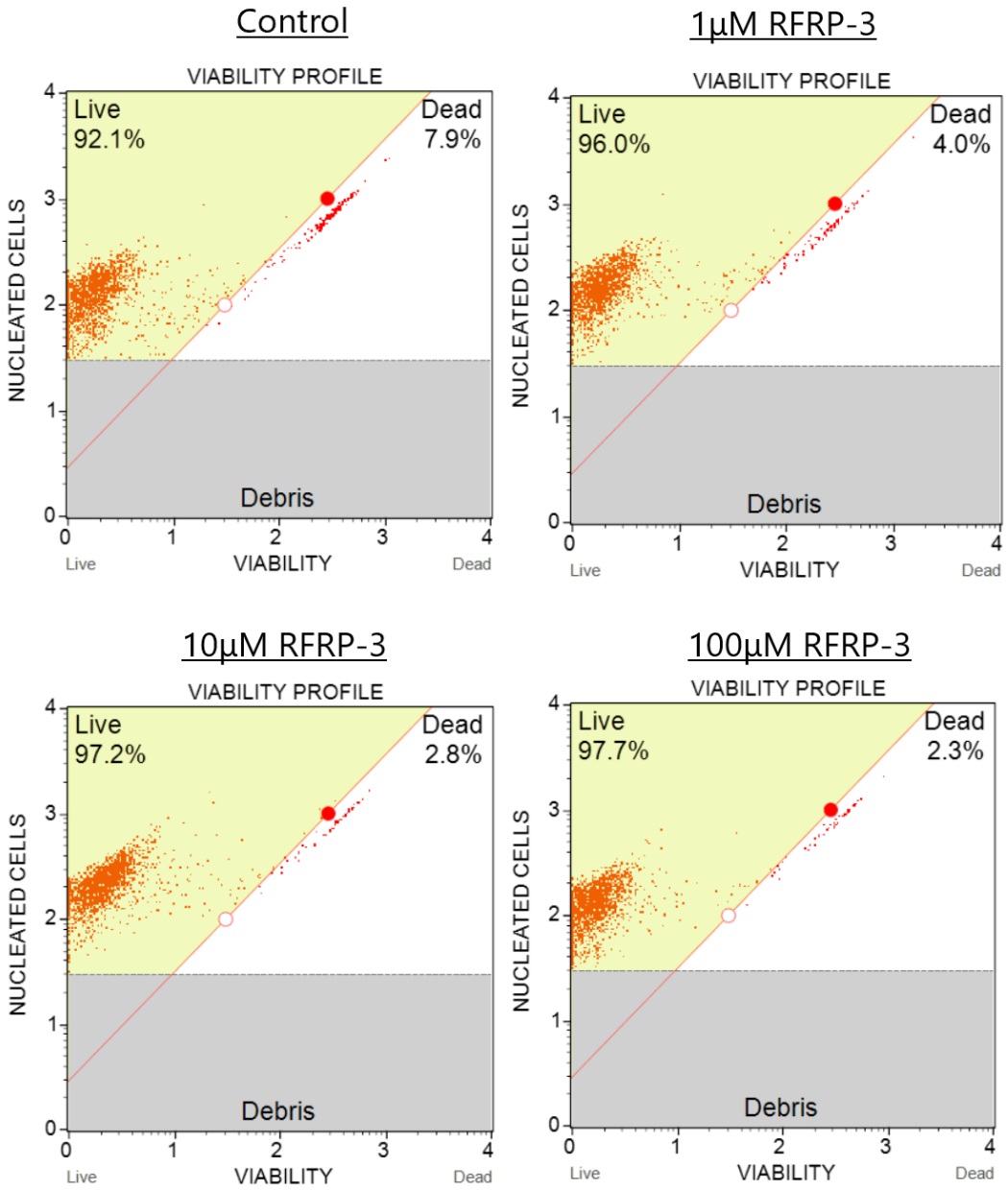



*Figure 12: Cell viability of LN18 cells treated with 1uM, 10uM and 100uM RFRP-3 measured using flow cytometry*

Our MTT assays stipulated the lack of any effect on the LN18's viability by RFRP-3. While MTT assay is a versatile homogenous assay method that is incredibly useful in high throughput screening, it has some limitations. MTT is converted into soluble formazan via mitochondrial reduction by viable cells, and thus are more reminiscent of the cell's rate of metabolism. MTT assay is also less sensitive when compared to other fluorescent or luminescent-based assay. Furthermore, results produced by MTT

assays are qualitative, which only allows for relative quantification. Attempts were made to obtain a set of quantitative data using flow cytometry. Cell viability of LN18 cells was investigated after treatment with 1uM, 10µM and 100µM of RFRP-3 for 48 hours (Figure 12). Results revealed that RFRP-3 had little effect on the viability of LN18 cells, with all treatments having similar viability as the control (Control, 92.1%; 1µM RFRP-3, 96%; 10µM RFRP-3, 97.2%, 100µM RFRP-3, 97.7%).

MTT assays are poor predictors of any changes to cellular proliferation rates due to its reliance on the cells' metabolic activity. Therefore, even with the lack of any significant changes in viabilities both in the MTT assays and the aforementioned flow cytometry data, a conclusive declaration cannot be made about the involvement of RFRP-3 in the proliferation of LN18 cells. The study of any involvement of RFRP-3 in the proliferation of LN18 cells can be confirmed with flow cytometry by determining the percentage of proliferating cells based on Ki67 expression, a notorious marker for tumour aggressiveness (198). We thus followed up the viability results by looking at the effect of RFRP-3 on the proliferation rate of LN18 cells done by staining the cells with Ki67 antibody and observing cells that are stained with Ki67 antibodies using flow cytometry. Our results suggested that RFRP-3 had little effect on the proliferation rate of LN18 cells as well (Figure 13). Cells treated with RFRP-3 had a similar percentage of cells expressing Ki67 protein when compared to the control regardless of the administered concentration (Control, 96.83%; 1µM RFRP-3, 96.65%; 10µM RFRP-3, 96.27%; 100µM RFRP-3, 97.17%). Overall, the flow cytometry results reaffirmed the results obtained in the cell viability assay, where RFRP-3 was found to have little influence on the proliferation or apoptosis of LN18 cells.

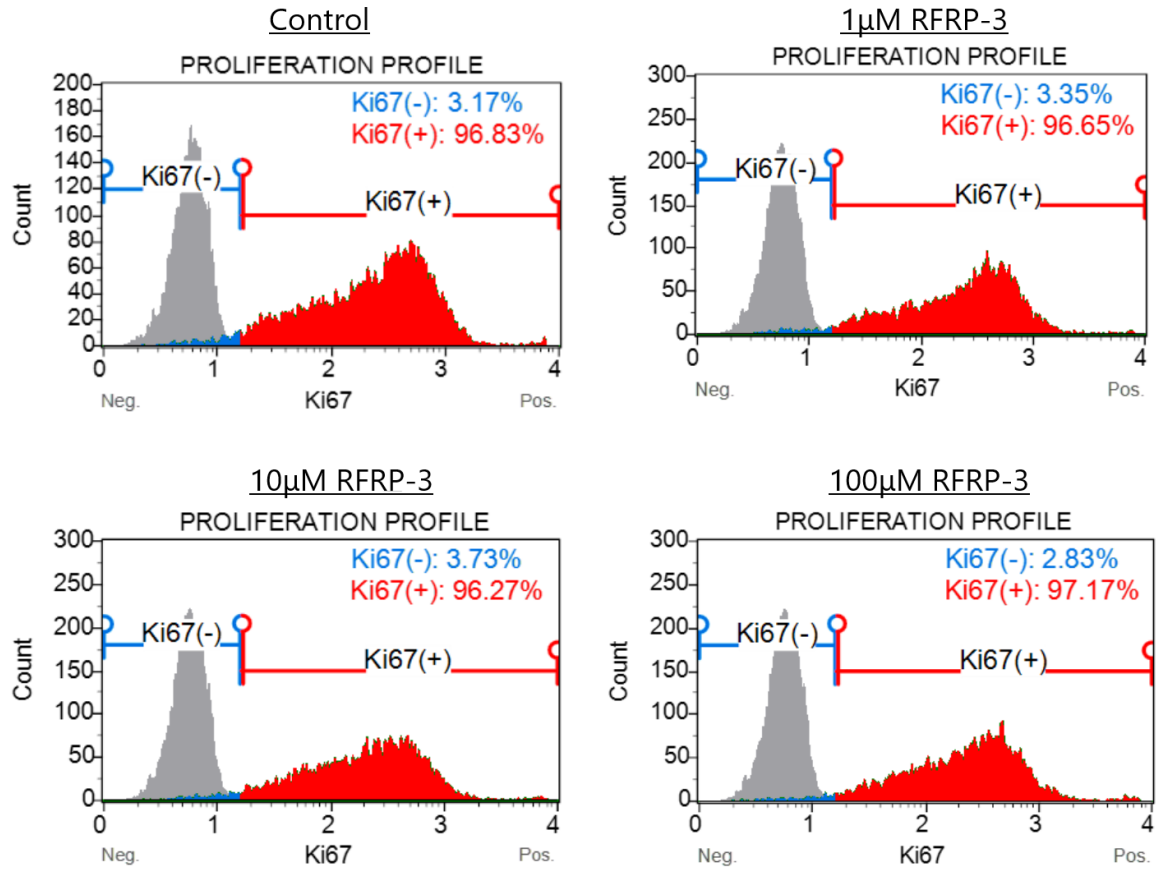



*Figure 13: Ki67 proliferation profile of LN18 cells after being treated with 1µM, 10µM and 100µM of RFRP-3, which was measured using flow cytometry.*

### 4.5 MGMT Gene Expression Test

GBM's sensitivity to TMZ is directly correlated to the expression level of MGMT, where A172 GBM cells' lack of MGMT expression due to promoter methylation yielded low TMZ $IC_{50}$ values (130). Consistent with previous reports, our expression tests reaffirmed the correlation between the sensitivity of GBM towards TMZ and the expression level of MGMT, where A172 had significantly lower expression of MGMT when compared to LN18 and T98 cells (Figure 14).

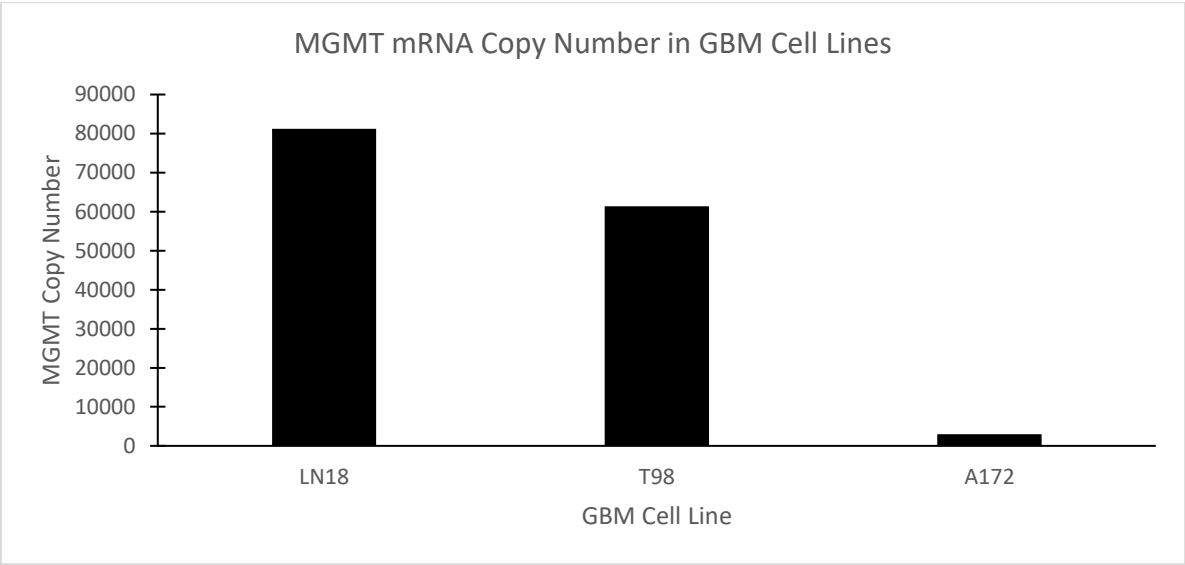



*Figure 14: MGMT mRNA copy number in three different GBM cell lines.*

Although RFRP-3 had no influence on the cell viability of LN18 cells, we nonetheless speculated the possibility of RFRP-3 and GPR147 playing a role in affecting the expression of MGMT in GBM. Since our previous result failed to demonstrate any difference in effect between the two RFRP peptides upon the cell viability of LN18, we decided to focus on RFRP-3 for the expression test since it is the functional homolog of avian GnIH. We decided to focus our efforts on three concentrations since none of the concentration tested in the RFRP treatment had any effect on the viability of LN18 cells. LN18 cells were treated with RFRP-3 (100nM, 1µM and 10µM) for 24 hours and 48 hours before the expression of MGMT was determined via Q-PCR.

Using GADPH as the internal control, the relative expression of MGMT in LN18 cells treated with RFRP-3 compared to the control group were determined. Treatment of LN18 cells with RFRP-3 did not produce any significant changes to the expression of MGMT genes when compared to the untreated

control (Figure 15A). The fold change of MGMT expression recorded was 1.14 (100nM), 1.05 (1µM) and 1.08 (10µM), respectively.

We then proceeded to expose the LN18 cells to RFRP-3 for 48 hours to investigate any effect of RFRP-3 on the expression of MGMT. Quantitative PCR measurements revealed that the relative MGMT mRNA expression in LN18 cells decreased upon exposure to RFRP-3 (Figure 15B). mRNA levels of MGMT in LN18 cells treated with 100nM (0.67) and 1µM (0.73) significantly decreased after 48 hours ($p < 0.001$), while no significant decrease was recorded for cells treated with 10µM (0.8) of RFRP-3 which was most likely caused by the large standard error mean (SEM) recorded.

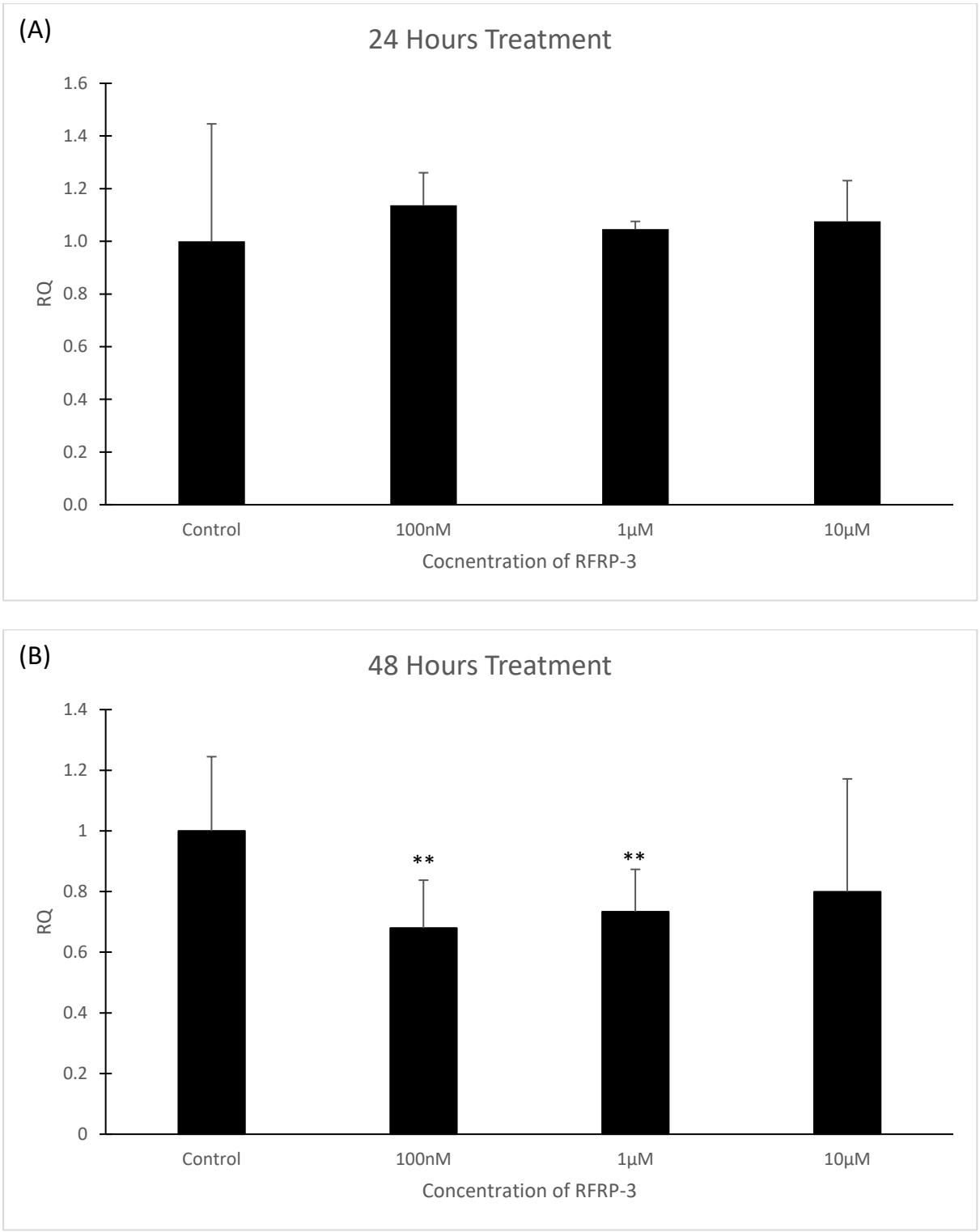



*Figure 15: (A) Measurement of MGMT gene expression in LN18 cells after being treated with RFRP-3 for 24 hours by quantitative PCR. (B) Measurement of MGMT gene expression in LN18 cells after being treated with RFRP-3 for 48 hours by quantitative PCR. LN18 cells treated with 100nM ($p < 0.001$) and 1µM ($p < 0.001$) experienced significant decrease in MGMT gene expression. All data are shown as the mean ± SEM from duplicated experiments. Independent sample t-test was performed between the treated cells and the control group. The presence of asterisks indicates a statistically significant result (** = $p < 0.01$).*

### 4.6 Concomitant Treatment of TMZ & RFRP-3

Our previous result indicated that MGMT expression was decreased upon treatment with RFRP-3 for 48 hours. Based on our Q-PCR results, it was hypothesised that TMZ potentiation in LN18 cells could be achieved upon treatment with RFRP-3. To confirm the hypothesis that RFRP-3 sensitises GBM cells by down-regulating MGMT expression, we examined the combinational effect of TMZ plus RFRP-3 in LN18 cells by dividing the cells into two treatment groups: one group being treated with TMZ, with the other group being treated with TMZ in the presence of RFRP-3.

In order to proceed with the 48-hour concomitant treatment of TMZ and RFRP-3, we first needed to determine the $IC_{50}$ of TMZ in LN18 cells after 48 hours. To achieve that goal, $5x10^3$ LN18 cells/well were seeded in 96 well plates and were subjected to a wide range of TMZ concentrations (125µM to 875µM with increments of 125µM) for 48 hours. The range of concentration of TMZ was chosen to cover a wide range of concentration for a more accurate representation of the $IC_{50}$ value in LN18 cells after 48 hours. Figure 16 shows the viability recorded for LN18 cells exposed to different concentrations for 48 hours. According to the data, the $IC_{50}$ for TMZ in a 48-hour treatment is 648µM.

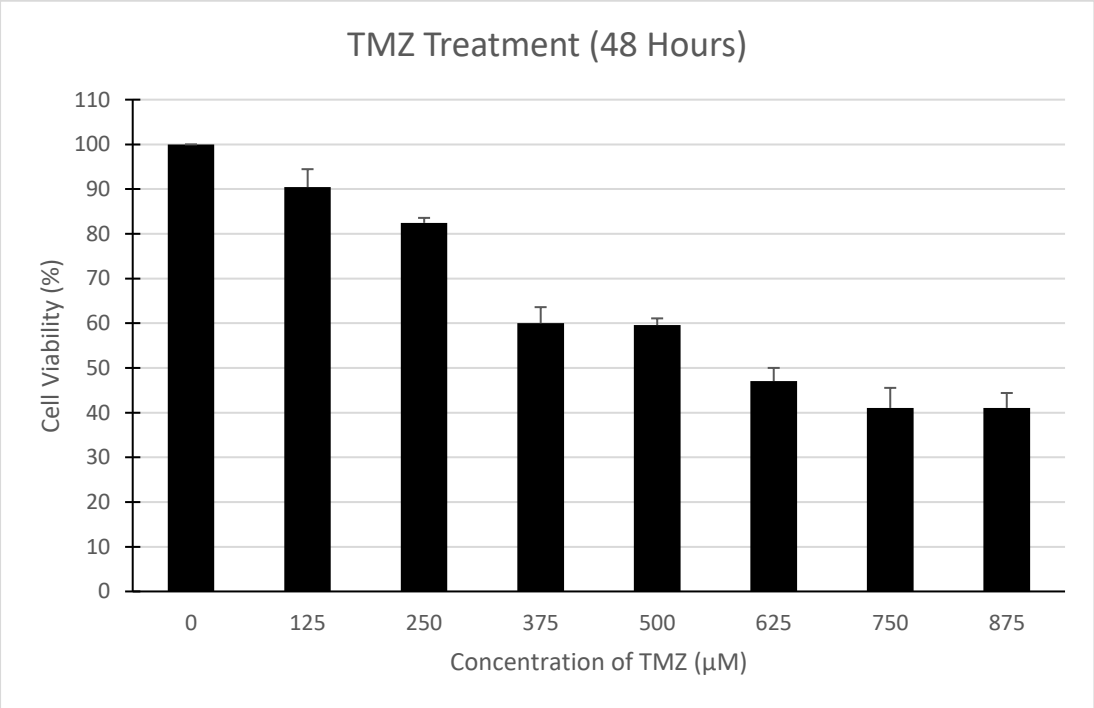



*Figure 16: Cell viability of LN18 cells subjected to a range of different TMZ concentrations. All data are displayed as the mean ± SEM from triplicated experiments.*

For the concomitant treatment of RFRP-3 and TMZ, three different concentrations of TMZ (125µM, 350µM and 700µM) and six different concentrations of RFRP-3 (1nM, 10nM, 100nM, 1µM, 10µM and 100µM) was used. 700µM of TMZ was used for the following concomitant treatment to account for any variance or errors in the $IC_{50}$ experiments. 125µM and 350µM of TMZ were also included to observe any dose-dependent changes. 100nM and 1µM of RFRP-3 conferred a significant decrease in MGMT expression in our previous experiment, but we ultimately decided to not only look at the effects of 100nM and 1µM of RFRP-3 when treated concomitantly with TMZ but also the full range of RFRP-3 concentrations from 1nM up to 100µM used in the MTT viability assays.

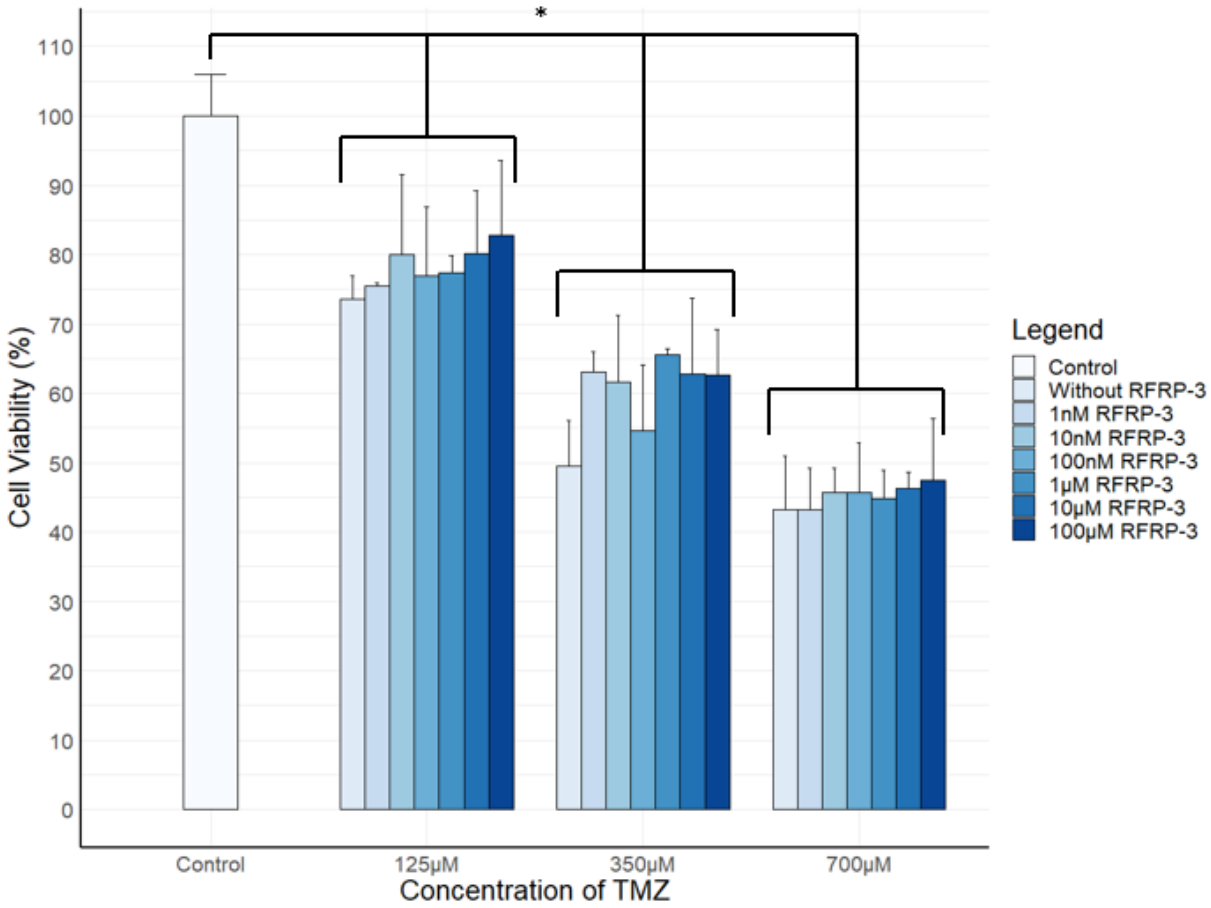



*Figure 17: Cell Viability of LN18 cells in two different treatment groups: TMZ only, TMZ plus RFRP-3 for 48 hours. Significant reduction in cell viability was induced by all concentrations of TMZ when compared to the control. No significant difference was detected between the two treatment groups. All data are displayed as the mean ± SEM. One-way ANOVA was performed between cells treated with TMZ only, cells treated with TMZ plus RFRP-3 and the control group. All data are shown as the mean ± SEM from triplicated experiments. The presence of an asterisk signifies a statistically significant result (* = $p < 0.05$).*

Administration of TMZ in LN18 cells for 48 hours predictably saw a dose-dependent decrease in the cell's viability (Figure 17). The decrease in viability across every tested treatment was significant in

relation to the control ($p<0.05$). In contrast to our MGMT gene expression test however, the presence of RFRP-3 did not induce any significant viability decrease regardless of the concentration of TMZ or the concentration of RFRP-3. The presence of RFRP-3 when LN18 cells were treated with 125µM,350µM or 700µM of TMZ either showed similar cell death or slightly decreased cell death. The presence of RFRP-3 when the cells were treated with 125µM of TMZ showed 1.9%, 6.4%, 3.4%, 3.8%, 6.6%, and 9.3% increase in viability compared to the single treatment arm respectively in the ascending order of RFRP-3 concentration. The combination treatment arm when the cells were treated with 350µM of TMZ saw the highest increase in viability compared. The combination treatment arm produced a 13.6%, 12%, 5.1%, 15.9%, 13.3% and 13.1% increase in viability compared to the single treatment arm, respectively. In the case of LN18 cells treated with 700µM of TMZ, the increase in viability were more modest, coming in at 0%, 2.5%, 2.5%, 1.6%, 3.1% and 4.2%, respectively. However, none of the combination treatment arms produced statistically significant changes when compared to its single treatment arm regardless of the concentration of TMZ. Taken together, these results point to the assertion that the addition of RFRP-3 does not reduce the resistance of LN18 cells towards TMZ, which greatly contrasted the results obtained in the gene expression test.

### 4.7 MGMT Gene Expression in the Presence of TMZ

The contrast in the results between the TMZ treatment and the initial MGMT expression test prompted us to look into the expression of MGMT in the presence of RFRP-3 in combination with TMZ. We decided to revisit the amount of time and the concentrations of RFRP-3 that provided a decrease in MGMT expression in the previous expression study. The concomitant treatment showed a dose-dependent decrease in viability when exposed to TMZ, but none of the concentrations used in conjunction with RFRP-3 showed a further decrease in cell viability that would reflect the results of the first expression study. Therefore, we decided to use the $IC_{50}$ concentration for the administration of TMZ in this study. LN18 cells were divided into three treatment groups (100nM and 1µM of RFRP-3 only, 700µM of TMZ only, and 700µM of TMZ in the presence of 100nM and 1µM of RFRP-3) and were treated for 48 hours (Figure 18).

The introduction of 700µM of TMZ significantly increased the expression of MGMT ($p = 0.01$), inducing a fold change of 2 when compared to the control. The administration of RFRP-3, however, did not decrease the expression of MGMT; it was found that the expression of MGMT was not affected in the presence of RFRP-3 at all. The fold change observed was 1.39 and 1.31 respectively for cells treated with 100nM and 1µM of RFRP-3, with the caveat that it was not statistically significant. While the

increase in MGMT expression was not statistically significant, the decrease in MGMT expression in our initial look at RFRP-3's effect on MGMT expression was not reflected here. The combination treatment arm for both 100nM and 1µM of RFRP-3 saw a decrease in MGMT expression when compared to its single TMZ treatment counterpart with fold changes of 1.7 and 1.2, respectively. However, the decrease in the combination treatment arms was statistically insignificant when compared to the single TMZ treatment arm. In essence, these results did not reflect those seen in the initial MGMT gene expression studies, as RFRP-3 did not seem to have any effect on the expression of MGMT.

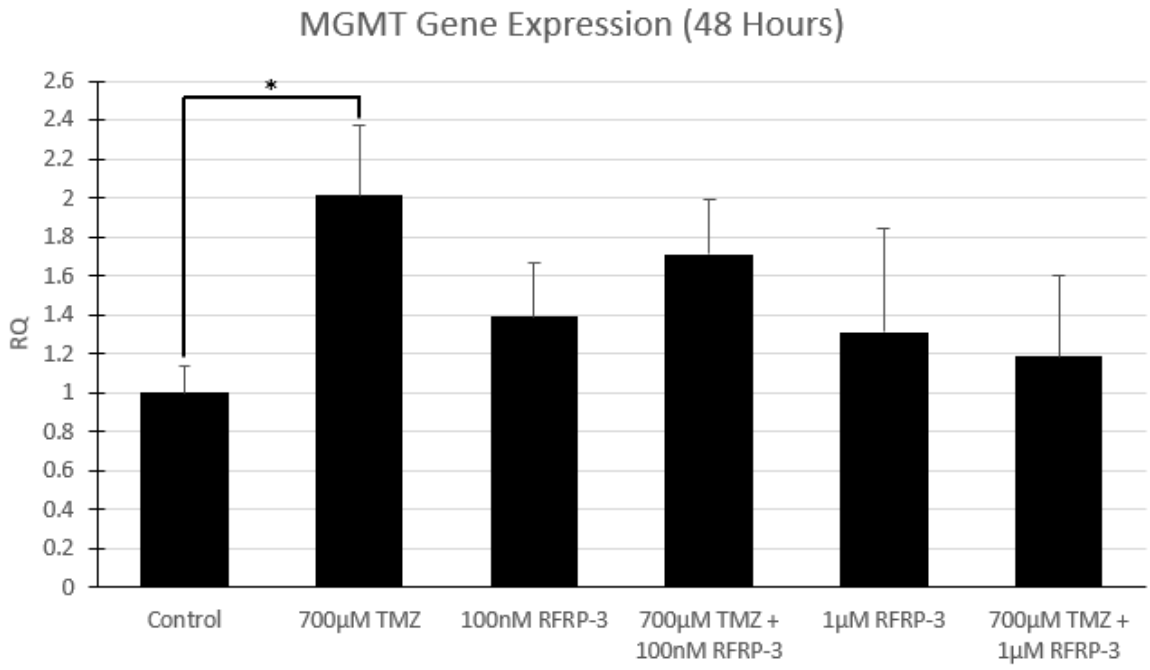



*Figure 18: Measurement of MGMT gene expression of LN18 cells in three different treatment groups: 700µM of TMZ only, 100nM and 1uM of RFRP-3 only, and 700µM TMZ in the presence of 100nM and 1µM of RFRP-3. LN18 cells treated with 700µM of TMZ increased the expression of MGMT significantly (p = 0.01) when compared to the control. No significant difference was produced between the three treatment arms, although it has to be noted that p-value between 700µM of TMZ alone and 700µM in the presence of 1µM of RFRP-3 was 0.068. It also has to be noted that 700µM of TMZ in the presence of 100nM of RFRP-3 had a p-value of 0.064 relative to the control. All data are displayed as the mean ± SEM from triplicated experiments. One-way ANOVA was performed between the treatment groups and the control group with the presence of an asterisk signifying a statistically significant result (* = p < 0.05).*

# 5. Discussion

## Summary of Findings

In the current study, we have shown that GPR147, the primary receptor for RFRP-3, was present in GBM cell lines resistant to TMZ while absent in a GBM cell line notably sensitive to TMZ at the proteomic level. We found that RFRP-3 had little to no effect on the viability of LN18 cells, signalling their lack of participation in the proliferation or apoptosis process in LN18 cells. This premise was further confirmed in our flow cytometry results where we showed that RFRP-3 not only had little effects on the viability of the cells, it also had no influence in the expression of proliferation marker Ki67, indicating its lack of involvement in the proliferation of LN18 cells. While our initial MGMT gene expression test revealed modulation of MGMT expression by RFRP-3, the result was not reflected in the concomitant treatment of LN18 cells with RFRP-3 and TMZ. Further gene expression analysis of LN18 cells in the presence of TMZ revealed RFRP-3 played no part in modulating the expression of RFRP-3, indicating a possible false positive with the initial result. All in all, we can conclusively pronounce that RFRP-3 has no role in affecting the viability of LN18 cells either via apoptosis or proliferation as well as and in the modulation of MGMT expression, making it an unsuitable candidate to potentiate the effects of TMZ.

## 5.1 Expression of GPR147 in Three Different GBM Cell Lines

The results from both the regular PCR and quantitative PCR demonstrated the expression of GPR147 in GBMs, albeit at a lower expression level as demonstrated by the 35 cycles of regular PCR required to produce GPR147 amplification and the low mRNA copy number produced in the Q-PCR. This result is consistent with the results of Feve *et al.*'s analysis of GPCR expression in GSCs and GBM such as the U87 cell line, where GPR147 expression had a Ct value that was larger than 31.5 in both cases (197). Critically, A172, a chemosensitive GBM cell line and thus susceptible to TMZ alkylation, had lower GPR147 expression than both LN18 and T98 cells. Furthermore, GPR147 proteins were not found in our Western Blot results despite the clear presence of its corresponding mRNA. The relationship between the expression of mRNA and its corresponding protein varies depending on the genes and its biological categories (199). Li *et al.*'s analysis suggested that mRNA levels can explain at least 56% of the variance in protein abundance in mammals, but not all of it (200). The expression of mRNA into protein could be affected by various post-transcriptional or translational modifications and regulations that could explain the difference in GPR147 expression in A172. Post-transcription modification is a complex process which involves processes such as $m^7G$-capping which predates other modifications such as internal $N^6A$ methylation, 3'-poly(A) addition, $N^1$-methyladenosine, and exon splicing. A172

cells may play an active role in modulating these post-transcriptional modifications which have been found to affect mRNA structural integrity and stability, maturity, promotion of mRNA translation, and the protein produced (201). Furthermore, the *in vivo* half-life of GPR147 in A172 cells may also be shorter than that of LN18 and T98 cells. A172 cells could be actively controlling the turnover of GPR147 receptors by controlling the rate of synthesis and the rate of degradation. Desensitization of GPCRs is achieved by phosphorylation of the receptor either via homologous desensitization by GPCR kinases or heterologous desensitization by second messenger-dependant protein kinases such as PKA and PKC (202). Desensitization of GPCRs promotes the binding of β-arrestins (β-arrestins 1 and 2) that leads to clathrin-mediated endocytosis of the receptor (203). Depending on the affinity in which the receptor binds to β-arrestin 2, the GPCR is either rapidly recycled back to the cell surface or be targeted for degradation (202). It is possible that GPR147 binds to β-arrestin 2 with the same affinity as β-arrestin 1, internalizing the stable GPR147-β-arrestins complex that is targeted by endosomes, leading to the endocytosis and downregulation of GPR147s (202). The lower expression of GPR147 mRNA coupled with the absence of GPR147 receptors led us to believe that GPR147 and RFRP may play a role in maintaining LN18 and T98 cells' resistance to TMZ by perhaps regulating the expression of MGMT.

## 5.2 cAMP Assay

Previous studies have shown that the signalling pathway initiated upon binding of RFRP-3 to GPR147 in the gonadotropic axis involved the activation of $G_{\alpha i}$. It has to be noted nonetheless that RFRP-3 could have activated a different signalling pathway like its target GnRH in a tumour microenvironment. As it stands, there is little to no research done in this area to suggest any division in RFRP-3 signalling between gonadotropic cells and tumour cells; thus we speculated that GPR147 follows the canonical pathway upon activation, binding to $G_{\alpha i}$ and subsequently inhibiting the actions of adenylate cyclase.

In our testing, A172 cells had no changes in the cAMP concentration after being exposed to RFRP-3. The finding was expected since our Western Blot results showed no GPR147 receptor expression. Although the results were insignificant, there was a slight reduction in cAMP concentration across all the treatments. While we have established the absence of GPR147 in A172 cells, RFRP-3 has been known to bind to the NPFF receptor, also known as GPR74 (204). Various RFRP and NPFF analogues were used by several groups of researchers to determine the binding affinity of both peptides to GPR147 and GPR74. It was found that RFRPs had had a binding affinity of about 100 times higher for GPR147 than NPFFs while NPFFs had a binding affinity to GPR74 of about ten times higher than RFRPs (205-208). NPFFs have a C-terminal motif of PQRFα which shares some similarity and resemblance to

RFRP-3's LPXRFa C-terminal motif. This is imperative since Elhabazi *et al.* showed in their binding experiments that the presence of the C-terminal motif allowed RFRP-3 to bind GPR74. RFRP-3 bound to GPR74 at a $K_i$ of 67nM as opposed to the $K_i$ of 0.2nM upon binding to GPR147 (209). GPR74, like GPR147 also binds to Gαi upon activation of the receptor, and chicken GnIH was found to modulate cAMP concentration slightly in COS-7 cells transfected with GPR74 (210). It is thus possible that RFRP-3 bound to GPR74 in A172 cells, decreasing the concentration of cAMP slightly despite the absence of GPR147. The expression status of GPR74 in A172 cells is unknown, although Feve *et al.* did detect the expression of GPR74 in U87 GBM cells (197).

Our results showed a dose-dependent reduction in cAMP assay upon administration of RFRP-3 in LN18 cells. This result postulates that the receptors expressed in GBM are active, and the receptor conveys a similar downstream signalling pathway as those found in the gonadotropic axis. Unfortunately, the results were not statistically significant; therefore, the previous statement could not be taken with absolute certainty. This could very well be due to the high individual variations in cAMP production within the cells between biological replicates. Furthermore, since Gαi-coupled GPCRs inhibits the production of cAMP, forskolin is commonly used to stimulate the production of cAMP in Gαi-coupled receptor assaying so that inhibition of AC can be easily observed. Due to limitations of the experimental conditions, forskolin was not used during the assaying of Gαi-coupled GPR147, which might explain the lack of significant results statistically despite the discernible trend. All the cells were starved overnight during the experiments which was a standard protocol in most cAMP assay to synchronise the cell cycles while decreasing the basal cAMP level, which made any reduction in cAMP level harder to detect.

### 5.3 Cell Viability Assay

RFRP-3 has shown the capability of inhibiting the MAPK/ERK pathway by suppressing the cAMP dependant ERK1/2 signalling in the pituitary (195). Our cAMP assay showed the decrease in cAMP concentration via $G_{\alpha i}$ upon RFRP-3 administration, and thus we hypothesised that it was followed by the inhibition of the MAPK/ERK pathway. The role of ERK in cancers are well documented, playing a critical role in promoting angiogenesis, uncontrolled cell proliferation and promoting resistance towards drug therapies in various cancers from hepatocellular carcinomas to prostate cancers, human thyroid cancers and more (211). We speculated on the ability of RFRP-3 to influence the growth of GBMs via the MAPK/ERK pathway; however, our data demonstrated the lack of any significant effect on the viability of LN18 cells. This suggested RFRP-3 had little or no effect on the apoptosis of GBM

cells. It is possible that while RFRP-3 did activate $G_{\alpha i}$ upon binding GPR147, its downstream signalling pathway in GBMs and cancer cells are different from that found in the pituitaries, similar to the phenomenon found in GnRH where its downstream signalling depended on its cellular environment. It also has to be noted that the regulatory targets of MAPK cascades are numerous, where its targets can derepress genes involved in proliferation, promote protein ubiquitination and degradation, promote cancer cell invasiveness and much more (212). Perhaps in this case, none of the transcriptional regulatory targets involved in cancer cell death was affected by the downregulation of the MAPK/ERK cascade.

Similarly, our data also point out the lack of any significant effect by RFRP-1 on LN18 cells' viability, suggesting the lack of any anti-proliferative or apoptotic effect like its RFRP-3 counterpart. This finding is inconsistent with the dynamics of RFRP-1 and RFRP-3 that are present in the pituitary, where RFRP-1 have been shown to have different effects than RFRP-3 despite the fact that both peptides bind to the same GPCR (193, 213). The difference between the two mature peptides may arise from the different posttranslational processing of the two peptides. Although RFRP-1 and RFRP-3 are derived from the same precursor, alternate processing of the two peptides may lead to different functions, as observed in several other peptides (192). It could also be the case that RFRP-3 and RFRP-1 trigger different conformational states upon binding with GPR147 that relay different signalling cascades in the pituitary (214). Instead of having different *in vivo* effects like in the pituitary, RFRP-1 and RFRP-3 could elicit the same effects and the same signalling mechanisms in a tumour environment. It is well known that GPCRs can possess more than one binding site, an orthosteric site which is the binding site of endogenous ligands and an allosteric site which is a separate binding site on the same receptor (215). Binding of allosteric ligands can alter the binding affinities of endogenous ligands to the orthosteric site, thus possibly changing the conformation of the receptor (215). The interaction between the allosteric site and the orthosteric site of GPR147 in a tumour environment may cause the receptor to activate similar downstream signalling cascades regardless of the binding of RFRP-1 or RFRP-3. However, it also cannot be ruled out that RFRP-3 and RFRP-1 may trigger different signalling cascades and effect in a tumour environment, but none of them exhibits apoptotic effects.

## 5.4 Flow Cytometry

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was first developed by Mosmann in the 1980s and has been the gold standard for *in vitro* cytotoxicity assays since (216). While MTT assay's versatility as a homogenous assay is widely used in drug screening, it

has several flaws and limitations. MTT assays are affected by various phytochemicals such as polyphenols (217), compounds generating superoxide such as nano-scale $TIO_2$ (218) and antioxidants (219) interfering with the results due to their intrinsic reductive potential nature. Since there is a huge dependence on the metabolic function of the cells to convert MTT into formazan products, there is also a correlation between the glucose concentration of the cell culture medium and the reductive rate of MTT (220). MTT reduction not only varied significantly between different tumour cell lines, but the reduction rate was also affected by the concentration of glucose, most notably the decrease in MTT reduction when using a glucose-free medium (220). These can cause MTT assay to either overestimate the cell viability or fail to detect any decrease in cell numbers. MTT assay is also highly sensitive to light, less sensitive than other fluorescent and luminescent methods and only provide qualitative data instead of a quantitative cell count (217). Furthermore, MTT assay showed higher variability in the linear range, which suggests compromised accuracy as well as diminished reproducibility (221).

Our MTT assays failed to produce any meaningful changes in LN18 cell viability and merely provided a qualitative set of data. Therefore, we aimed to get a firmer grasp about RFRP-3's role in GBM from a more quantitative source. The fluidics within the flow cytometry allows cells suspended in a fluid to pass through a laser beam one cell at a time, using the scattered light to differentiate between the cells and their components. Flow cytometry allows for more quantitative and accurate measurements of the cell viability after being treated with RFRP-3. The results from our flow cytometry experiment reaffirmed our MTT assay results. None of the treatments recorded cell viabilities lower than 90% while only having slightly lower viability than the control. This set of results cemented the fact that RFRP-3 does not play any role in inducing apoptosis in GBM cells.

While all the previous experiments showed the lack of any effect of RFRP-3 on the viability of LN18 cells, we could not concretely declare the role of RFRP-3 in the proliferation of LN18 cells. As previously mentioned, MTT assays rely on the metabolic reductions of MTT into formazan by the cells. Since the metabolic activity are innately tied to the growth phase and cell cycle of the cells, the need for MTT reduction is a varying parameter that leads to inaccurate reflection cellular proliferation rates (222). An instance was found where MTT assay severely underestimated the anti-proliferative effect in the treatment of human prostate adenocarcinoma cells when compared to more conventional proliferation assays such as CyQUANT proliferation assays that involve the measurement of DNAs (223). This prompted us to investigate any anti-proliferative effect that may be exerted by the

administration of RFRP-3 via flow cytometry. Ki67 is a notorious proliferation marker protein, found mainly within the nucleus. Ki67 is highly expressed in proliferating cells from the $G_1$ to M phase of the cell cycle but is absent in resting cells (G0) (224). A sharp decrease in Ki67 also happens in the later stages of mitosis, such as anaphase and telophase (225). Naturally, Ki67 becomes associated with the proliferative activity of cells and thus is an infamous marker of the aggressive intrinsic cell populations in malignant tumours, constituting a valuable prognostic factor in gliomas (225, 226). To find out any anti-proliferative effect exerted by RFRP-3, we investigated the expression of Ki67 using flow cytometry by staining the cells with Ki67 antibody. Unfortunately, all our treatments had a similar percentage of LN18 cells expressing Ki67 regardless of the concentration of RFRP-3 introduced, indicating that RFRP-3 does not reduce the proliferation of GBM cells.

### 5.5 MGMT Gene Expression Test

One of the major hurdles in the treatment of GBM is the development of resistance to chemotherapeutics such as TMZ, where MGMT is often relied upon by GBMs to confer resistance against TMZ. We postulated on the possibility of RFRP-3 affecting the expression of MGMT based on the lack of GPR147 receptors in the chemosensitive A172 cell line. Our data demonstrated a time-dependent decrease in the expression of MGMT in LN18 cells upon administration of RFRP-3. While 24 hours of treatment had no effect on MGMT expression, the 48-hour treatments clearly showed a significant decrease in MGMT gene expression within LN18 cells. The results from this study potentially point towards the ability of RFRP-3 to sensitise TMZ resistant GBM cells. To confirm whether RFRP-3 plays a role in sensitising GBM cells towards TMZ, co-treatment of RFRP-3 together with TMZ is essential. If concomitant treatment of TMZ and RFRP-3 induces higher cytotoxicity than just TMZ alone, it will then justify our assumption that RFRP-3 reduces the resistance of GBM cells towards TMZ.

Various transcription factors were found to be responsible for the activation of MGMT transcription. AP-1, a transcription factor mediated by the PKC signalling pathway, was found to have matching consensus sequences that act as a binding site within the MGMT promoter and is responsible in regulating the activity of MGMT promoter (227, 228). NF-κB/p65 is another transcription factor that was found to be involved in the regulation of MGMT expression, where administration of TMZ was found to increase the transcriptional activity of NF-κB (229). Song *et al.* also discovered the disruption of the NF-κB signalling by fluoxetine was able to inhibit MGMT expression in glioma cells (230). NF-κB is notorious as a family of transcription factors involved in regulating the expression of multiple target

genes and it regulates MGMT expression by interacting with the two putative NF-κB sites in the MGMT promoter region (231). Other transcription factors such as SP1, HIF1α and p300 were also found to be involved in the transcription of MGMT in GBMs (232).

As mentioned above, the RFRP-3-GPR147 downstream signalling cascade has the ability to inhibit the phosphorylation of ERK in the MAPK pathway. The MEK-ERK signalling pathway has also been deemed to be responsible for dictating the expression of MGMT (233). Sato *et al.* revealed MEK inhibition corresponded with the downregulation of MGMT expression in stem-like GBM cells. p53 was found to be capable of inhibiting MGMT expression (233), and Sato *et al.* noted in their studies that MEK inhibition corresponded to the downregulation of MDM2, a negative regulator of p53 that ultimately lead to the upregulation of p53 (233). MDM2 has also been found to induce the degradation of p300 that specifically activates and acetylates p53 in other studies. (234, 235). The MAPK pathway is made up of a series of serine-threonine kinases that mediate different intracellular signalling pathways. The c-Jun N-terminal kinase (JNK) pathway is amongst the MAPK signalling cascade and has also been found to play a role in contributing to GBM's chemoresistance towards TMZ (236). Several other pathways that have also been found to regulate MGMT expression in GBMs such as the EGFR/PI3K/Akt/mTORC1/2 pathway as well as the Wnt and Sonic Hedgehog (SHH) pathways (Figure 19) (237-239). With multiple signalling pathways responsible for the regulation of MGMT expression, it remains to be seen how RFRP-3 downregulates the expression of MGMT, making further analysis of RFRP-3's signalling mechanism essential if RFRP-3 does play a role in sensitising GBM cells towards TMZ.

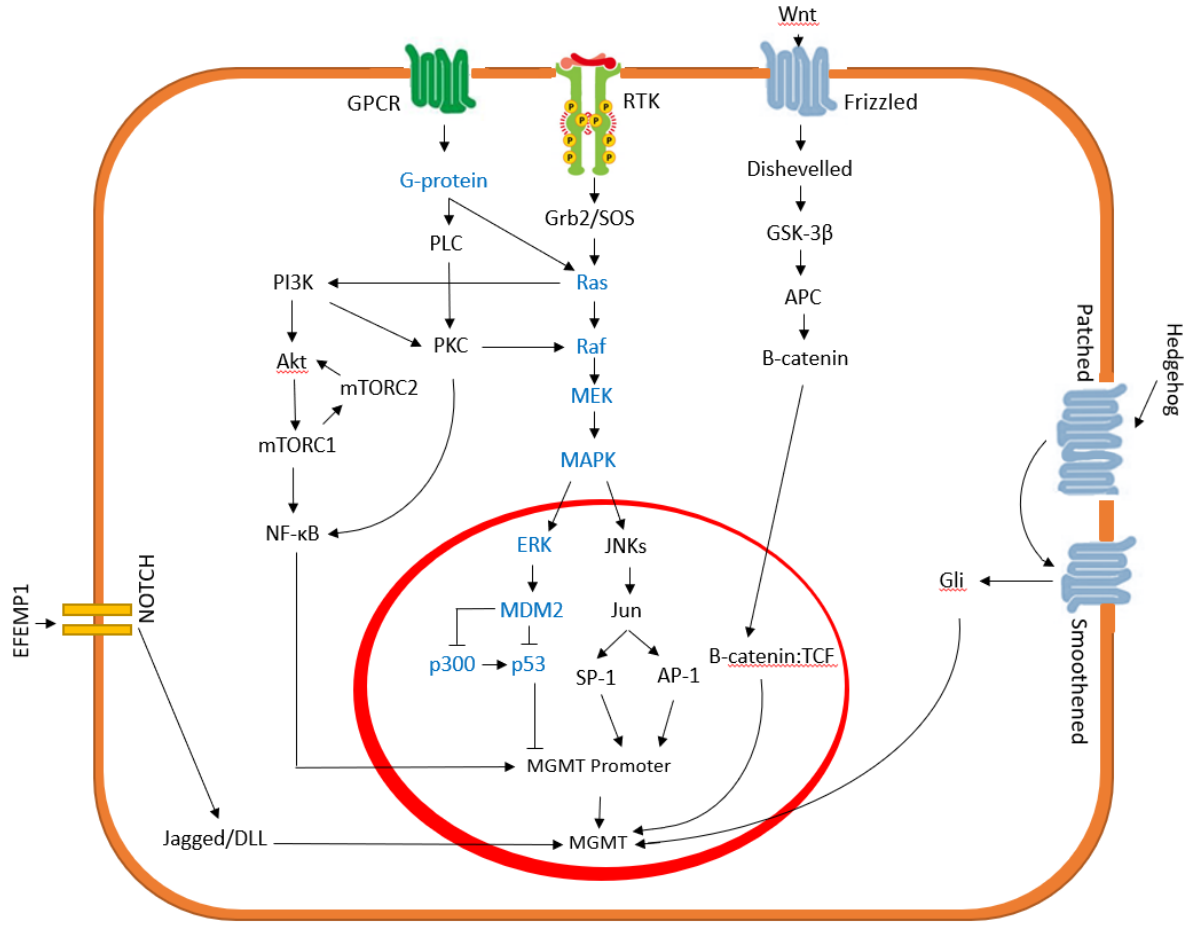



*Figure 19: Potential signalling pathways that have been found to modulate MGMT expression in GBM. The red circle indicates the nucleus. The PI3k/mTORC1/2 pathway activated by RTKs has also been stipulated to decrease the effectiveness of TMZ by upregulating MGMT expression that eventually leads to the activation of NF-κB (237, 240). It was also found that PKC acts as a critical intermediate in the signalling between EGFR and mTORC1 (241). Recently, the Wnt signalling pathway and Sonic Hedgehog (SHH) signalling pathway were discovered to play a role in inducing MGMT expression (238, 239). NOTCH-mediated chemoresistance via the upregulation of MGMT expression as well (242). The highest probable pathway relayed by the activation of RFRP-3 in the regulation of MGMT expression is highlighted in blue*

## 5.6 Concomitant Treatment of TMZ & RFRP-3

Glioblastoma is the most prevalent tumour among adults and is one of the most rapidly growing intracranial neoplasm. Established back in 2005, standard therapy against GBM constitutes surgical resection followed by adjuvant radiotherapy and TMZ treatment. TMZ is one of the main components against GBM, and the sensitivity of GBM against TMZ depends on the expression of MGMT. However, GBM patients are mostly resistant to the cytotoxic effects of TMZ due to the high expression of MGMT. Therefore, there is a need to investigate new therapeutic approaches to prevent unwanted DNA repair in GBM treatment

Our previous results suggested the possibility of RFRP-3 potentiating the effect of TMZ by down-regulating MGMT gene expression, hinting at the prospect of RFRP-3 being a viable therapeutic strategy against GBM. Peptides are on the rise as a therapeutic strategy for glioblastoma with the potential of more specific targeting and fewer side effects than conventional cancer therapies. Previous studies have intimated that RFRP-3 is unable to cross the BBB (243), but peptides can be easily modified chemically to improve pharmacokinetics. Cell-penetrating peptides such as those derived from human immunodeficiency virus type1 transcriptional activator TAT protein can be conjugated onto RFRP-3 to facilitate its transport across the BBB (244). RFRP-3 could also be modified and encased within nanocarriers for efficient transport across the brain.

To validate our previous assumption, we applied a concomitant treatment of TMZ and RFRP-3 and observed the cytotoxicity induced by both compounds using MTT assay. Our $IC_{50}$ results indicated that the concentration required for 50% growth inhibition in LN18 cells after 48 hours was 648µM. This result is pretty consistent with previous studies where LN18 was reported to have an $IC_{50}$ value of 774µM after 72 hours of treatment (245). However, contrary to our initial assumption, administration of RFRP-3 together with TMZ did not produce any significant increase in cell death after 48 hours. The lack of any significant increase in cell death regardless of TMZ concentration in the presence of RFRP-3 is hugely unexpected when compared to other studies. Other studies that identified compounds that reduced MGMT expression had a much more drastic synergistic outcome when treated together with TMZ, typically causing more than 20% to 50% increase in cell death than TMZ alone (230, 246, 247). The results of the treatment seeded doubts into our previous results, as the results obtained did not portray RFRP-3 as an agent capable of sensitising GBM cells towards TMZ by downregulating MGMT expression. The increase in RFRP-3 concentration does not correlate with potentiation of TMZ in LN18 cells either.

### 5.7 MGMT Gene Expression in the Presence of TMZ

Our previous expression tests revealed that MGMT expression was reduced by a fraction after 48 hours. In contrast, the concomitant TMZ treatment where none of the results showed any significant increase in cell death when cells were treated with TMZ in conjunction with RFRP-3 when compared to TMZ alone cast doubts into our initial results. We thus followed up the TMZ treatment with another expression test by dividing LN18 cells into three different treatment groups: RFRP-3 only, TMZ only and RFRP-3 plus TMZ.

Our *in vitro* results displayed a significant increase in MGMT mRNA expression after exposure to TMZ for 48 hours. Previous studies observing the expression of MGMT in the presence of TMZ using tumour tissues reported depletion of initial MGMT levels within hours of TMZ treatment which remained low for more than 24 hours (248, 249). In contrast to those observations, MGMT mRNA expression levels were increased in MCF breast cancer cells upon exposure of TMZ (250). Studies using rodents and human hepatoma cell lines also revealed a significant upregulation in MGMT mRNA and protein levels after exposure to DNA damaging agents (251-253). This was backed up by the results of Kitange's group where they revealed MGMT induction at the genomic and proteomic level after TMZ treatment in GBM culture cells as well as GBM xenografts (254). The cumulative available data reaffirms that GBMs cells upregulate the expression of MGMT as a defence mechanism against the DNA-damaging actions of TMZ.

In contrast to our previous expression test results, RFRP-3 failed to affect the expression of MGMT in any meaningful manner. Regardless of the concentration, RFRP-3 did not seem to downregulate the expression of MGMT mRNA as previously suggested. RFRP-3 also did not affect the expression of MGMT, even in the presence of TMZ. The initial lack of significant results in the concomitant treatments relative to the control seemed to suggest that RFRP-3 decreased any TMZ induced MGMT expression, although the concomitant treatment of TMZ and 100nM of RFRP-3 produced a p-value of 0.064 with respect to the control. However, the lack of significant results between the single treatment arm and the concomitant treatment arms seemed to suggest otherwise. The accruing results of this expression tests and the TMZ treatments seem to argue that the previous expression tests were false positives and RFRP-3 plays no part in the expression of MGMT. This explains the lack of synchronicity between the TMZ treatment and the previous expression test. Due to my negligence and lack of knowledge, the previous cell culture experienced a massive amount of passages, upwards to 60 passages. It is highly probable that the cells may have experienced unwanted mutations and epigenetic alterations throughout that period, providing us with false positives even though the previous MGMT expression test was repeated several times.

## 6. Future Directions

In this study, we showed that RFRP-3 most probably relayed the binding of RFRP-3 to GPR147 through the activation of $G_{\alpha i}$, decreasing the concentration of cAMP in LN18 cells. However, the transmission of molecular signals further downstream upon GPR147 activation was not discovered due to time constraints. Were RFRP-3 GPR147 to retain its cell signalling like those observed in the pituitary (195), it could inhibit the activation of PKA and ERK. Looking into the possible inhibition of PKA and ERK could go a long way in narrowing down the role of RFRP-3 in GBMs.

This study also affirmed that RFRP-3 played no role in the proliferation, apoptosis, or the regulation of MGMT expression in GBM cells. However, further studies could look at any possible effect of RFRP-3 in the migration and invasion of LN18 cells. VEGF and MMP-9 are important regulators of the angiogenic process, and the activation of VEGF and MMP-9 often hinges on the activity of the MAPK/ERK pathway (255-257). Looking into the effect of RFRP-3 on the migration or the angiogenic process of GBM after establishing the possible inhibition of PKA and ERK could be a feasible and fruitful route. An *in vitro* scratch assay would be the perfect preliminary test to evaluate the effect of RFRP-3 on cell migration.

It is vital to study the interaction between RFRP-3 and GnRH in a tumour cell environment. In stark contrast to RFRP-3, the role of GnRH in cancer cells have been more prominently explored, being found to either reduce proliferation or induce apoptosis in various types of cancers. Its signalling pathway in cancers has also been more thoroughly investigated. GnRH receptors were found to be present in U87GM and U373 GBM cells (258). Furthermore, the introduction of GnRH agonists was found to reduce the proliferation of GBM cells by up to 42.5% relative to the control, binding to $G_{\alpha i}$ in the process (258). In the pituitary, RFRP-3 is a prominent regulator of GnRH activity, actively suppressing the activity of GnRH and the release of gonadotropins. If RFRP-3 reprises its regulatory role on GnRH even in cancer cells, then identifying any interactions between RFRP-3 and GnRH could be the gateway into uncovering the role of RFRP-3 in GBM.

Furthermore, one must bring forth the fact that this study was conducted entirely on a 2D-based cell culture, and thus did not fully represent the heterogeneity of GBMs that are very well documented, where GBM tumour clusters form different niches. GBM tumour masses exist as a multi-layered entity in which different characteristics are found within each layer (259). The spatial distribution of the

heterogeneous GBM cells is dictated by the hypoxic gradient (260). The GBM tumour mass is made up of three layers specifically, the peripheral layer, intermediate layer, and the core. The core forms the inner hypoxic portion of the GBM mass that is highly enriched with GSCs where most of GBM's aggressiveness is said to stem from. The hypoxic gradient of GBM's tumour mass causes the GSC to be highly enriched in the core, remaining undifferentiated with high proliferative capabilities, with hypoxia-inducible factor-1α (HIF-1α) at the forefront of maintaining the hypoxic nature of the core. Studies have found that GBM cells cultured in a 3D environment possess a higher resistance towards TMZ that was potentiated by the hypoxia, with the majority of the GSCs in the core expressing MGMT (260-262). Persano *et. al* specifically discovered the influence of the stability of HIF-1α on the expression of MGMT, where the downregulation of HIF-1α was correlated to the downregulation of MGMT (262). Unlike a 3D environment where hypoxia is ever-present, *in vitro* 2D cultures have direct and unlimited access to oxygen and nutrients and naturally leads to the absence of interactions between the cells itself and the extracellular environment. It would be interesting to note any differences in effect that could be exerted by RFRP-3 in a 3D GBM culture more akin to its *in vivo* counterpart. Perhaps RFRP-3 is able to modulate the expression of MGMT by affecting the stability of HIF-1α in a low oxygen state, a factor that was not considered in this study.

As previously mentioned, the effect of cell migration was not probed in this study. An *in vitro* scratch assay represents the perfect preliminary test to evaluate RFRP's capability in affecting the migration of GBM cells. Since 2D monolayer cultures are cultured on a plastic substrate, this naturally lends to the absence of interactions between the cells itself and between the cells and the extracellular matrix (ECM). The normal brain ECM contains high levels of glycosaminoglycan, hyaluronan, proteoglycans and low levels of fibrous proteins such as collagen, fibronectin and laminin (263). GBMs have been known to alter the ECM composition by increasing the generation of fibrous proteins (264). Zhu *et al.* noted in their studies that the stiffness of the ECM matrix affected the invasive property of GBM cells in a 3D model, with a stiffer matrix correlating to lower expression of MMP proteins and vice versa (265). This was backed up by Kaphle *et al.* where they noted a decreased invasive capability of U87 cells with a decreased expression in MMP-2, MMP-9, tPA, and uPA expressions in cross-linked collagen hydrogels (stiffer matrix). It would be interesting to probe the effects of RFRP-3 in a 3D cell culture model to observe if it affects the matrix composition in the form of collagen of hyaluronan in addition to any effects on the expression of angiogenic-related genes such as VEGF and MMP-9.

Lastly, it has to be acknowledged that while *in vitro* culture systems are widely used in preclinical trials owing to its widespread availability and ease of observation, tumour cells do not exist in a vacuum *in vivo*. The extracellular matrix and the environmental factors play a huge role in sustaining proliferative signalling in cancers, inducing angiogenesis, promoting genomic and instability, promoting inflammation etc (266). In any future studies, environmental factors, which was not considered during this study could be incorporated to observe the interactions with administered RFRP-3. For example, steroid hormones that play a key role in brain development and differentiation have been found to affect GBM growth (267). Estrogen receptors are found in glial cells and GBM cells, and estrogens and the actions of tamoxifen were discovered to play a role in inducing apoptosis in GBM cells (267). Female nude rats implanted with GBM cells had higher survival rates than male rats (268). This was augmented by the fact that male and female rats treated with estrogen posted better survivals than ovariectomised, untreated and progesterone-treated rats (268), which might explain the differences in GBM incidences between male and female as well. Therefore, incorporating environmental factors such as steroid hormones could be a viable alternative going forward to study and scrutinise any possible synergistic effects between RFRP-3 and any incorporated environmental factors.

## 7.Timeline

| Project Activities | Year One | | | | | | | | | | | | | Year Two | | | | | | | | | | | Progress |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2018 | | 2019 | | | | | | | | | | | 2020 | | | | | | | | | | | |
| | N | D | J | F | M | A | M | J | J | A | S | O | N | D | J | F | M | A | M | J | J | A | S | O | N | |
| Literature Review | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | | | 100% |
| Determination of GPR147 Expression in LN18 | • | • | • | | | | | | | | | | | | | | | | | | | | | | 100% |
| Determination of GPR147 Expression in A147 and T98 cell line | | • | • | | | | | | | | | | | | | | | | | | | | | | 100% |
| Determination of GPR147 at the proteomic level (Western Blot) | | • | • | | | | | | | | | | | | | | | | | | | | | | 100% |
| LN18 RFRP Treatment | | | • | • | • | • | • | • | | • | • | | | | | | • | | | • | • | | | | 100% |
| MGMT Gene Expression Test | | | | | | • | • | • | | • | • | • | | • | • | | | | | | | | | | 100% |
| Determination of cAMP expression in LN18 & A172 after GnIH treatment | | | | | | • | • | • | • | • | • | • | • | • | • | • | • | | | | | | | | 100% |
| Co-treatment of LN18 Cells with TMZ and RFRP-3 | | | | | | | | | | • | • | • | • | • | • | | | | | | • | • | • | | 100% |
| Flow Cytometry | | | | | | | | | | | | | | • | • | • | • | | | | | | | | 100% |
| MGMT Gene Expression Test in the Presence of TMZ | | | | | | | | | | | | | • | • | • | | | | | | | | • | • | • | 100% |
| Thesis Preparation | | | | | | | | | | | | | | | | | | | | | | | | • | • | 100% |
| Confirmation of Candidature (11/7/2019) | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Progress Review (13/3/2020) | | | | | | | | | | | | | | | | | | | | | | | | | | |
| Thesis Submission (11/11/2020) | | | | | | | | | | | | | | | | | | | | | | | | | | |

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