Relationship between MGMT and Thioredoxin Reductase 1: Implications in response to PRIMA-1MET (APR-246) and the design of novel targeting strategies in Glioblastoma

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Abstract

Glioblastoma Multiforme (GBM) is the most common and advanced form of astrocytic primary brain malignancy in adults. The prognosis remains poor, despite aggressive treatment with surgery, radiation and chemotherapy using the alkylating agent, Temozolomide (TMZ). The DNA repair protein, O6-methylguanine-DNA-methyltransferase (MGMT) interferes with TMZ cytotoxicity. High frequency missense mutations of the *TP53* tumor suppressor gene confer oncogenic mutant (mut) p53 activities, such as resistance to chemoradiation.

A previous study from our lab demonstrated the cytotoxic effects of PRIMA-1MET (APR-246), a small molecule designed to restore wild-type function to mutp53 and shed light into its differential effects based on MGMT expression irrespective of p53 status. In this study, we investigated the underlying mechanisms for p53-independent effects and MGMT-based differences in PRIMA-1MET-induced cytotoxicity in GBM. We hypothesized that PRIMA-1MET may mediate its p53-independent effects through modulation of redox balance and that this balance is altered based on levels of MGMT.

Using GBM cell lines isogenic for MGMT and with a different p53 status, we showed that PRIMA-1MET induced reactive oxygen species (ROS) and decreased thioredoxin reductase 1 (TrxR1) expression levels irrespective of p53 status. We further validated the importance of ROS induction for PRIMA-1MET cytotoxicity using pharmacological agents that modulate ROS levels such as ROS scavenger, N-acetylcysteine and ROS inducer, L-Buthionine-Sulfoximine which significantly affected the anti-proliferative effects of PRIMA-1MET. Strikingly, we provide evidence for a potential novel relationship between MGMT and TrxR1. Isogenic cell lines with high MGMT expression exhibited high expression of TrxR1, low levels of ROS while their counterparts exhibited low TrxR1 expression and high ROS levels. This relationship is confirmed following treatment with O6-Benzylguanine, an MGMT inhibitor and Auranofin, a well-characterized TrxR1 inhibitor, FDA-approved for rheumatoid arthritis.

Despite a few limitations in this study, including the small number of cell lines, we provide a hypothetical model to explain the differential effects of PRIMA-1MET-induced cytotoxicity based on MGMT expression. Additional experiments are required to grasp the clinical relevance of these new findings, specifically for repurposing Auranofin as a new TrxR1-targeting strategy to use in combination with PRIMA-1MET. These findings will ultimately lead

to the development of efficient treatment strategies to improve the dismal outcome of patients diagnosed with GBM.

Résumé

Le glioblastome multiforme (GBM) est la forme la plus courante et la plus avancée d'astrocytome primaire cérébrale malin chez l'adulte. Le pronostic reste mauvais, malgré un traitement agressif par chirurgie, radiothérapie et chimiothérapie avec un agent alkylant, le Témozolomide (TMZ). La protéine de réparation de l'ADN, l'O6-méthylguanine-ADN-méthyltransférase (MGMT), interfère avec la cytotoxicité du TMZ. Des mutations faux-sens à haute fréquence du gène suppresseur de tumeur *TP53* confèrent des activités mutantes oncogéniques (mut) p53, telles que la résistance à la chimioradiothérapie.

Une étude précedente de notre laboratoire a démontré les effets cytotoxiques de PRIMA-1MET (APR-246), une petite molécule conçue pour restaurer la fonction wtp53 de mutp53, et a mis en évidence ses effets différentiels basés sur l'expression de MGMT indépendamment du statut de p53. Dans cette étude, nous avons analysé les mécanismes sous-jacents pour les effets indépendants de p53 et les différences basées sur MGMT en relation avec les effets inhibiteurs de prolifération induits par PRIMA-1MET. Nous avons émis l'hypothèse que PRIMA-1MET peut médire ses effets indépendants de p53 grâce à la modulation de l'équilibre redox et que cet équilibre est modifié en fonction des niveaux de MGMT.

En utilisant des lignées cellulaires GBM isogéniques pour MGMT et avec un statut p53 différent, nous avons montré que PRIMA-1MET induit des espèces réactives de l'oxygène (ROS) et réduit l'expression de la thiorédoxine réductase 1 (TrxR1) indépendamment du statut de p53. Nous avons validé l'importance de l'induction ROS pour la cytotoxicité PRIMA-1MET en utilisant des agents pharmacologiques qui modulent les niveaux de ROS tels que ROS « scavenger », N-acétylcystéine et ROS inducteur, L-Buthionine-Sulfoximine qui affectent significativement les effets antiprolifératifs de PRIMA-1MET. Nous avons mis en évidence des preuves d'une nouvelle relation potentielle entre MGMT et balance redox. Les lignées cellulaires isogéniques ayant une expression MGMT élevée ont présenté une expression élevée de TrxR1, de faibles taux de ROS alors que leurs homologues ont présenté une faible expression de TrxR1 et des niveaux élevés de ROS. Cette relation est confirmée à la suite d'un traitement par O6-Benzylguanine, un inhibiteur MGMT et Auranofin, un inhibiteur de TrxR1 bien caractérisé et approuvé par le FDA pour l'arthrite rhumatoïde.

Malgré quelques limites dans cette étude, y compris le petit nombre de lignées cellulaires, nous fournissons un modèle hypothétique pour expliquer les effets différentiels de la cytotoxicité induite par PRIMA-1MET basé sur l'expression de MGMT. Des expériences supplémentaires sont nécessaires pour mieux comprendre ces nouvelles découvertes et leur pertinence clinique, en particulier pour réorienter Auranofin en tant que nouvelle stratégie de ciblage de TrxR1 à utiliser en combinaison avec PRIMA-1MET. Ces résultats peuvent finalement conduire à l'utilisation de stratégies de traitement efficaces pour améliorer le mauvais pronostic des patients diagnostiqués avec GBM.

Acknowledgements

I would like to first express my deepest appreciation to my supervisor, Dr. Siham Sabri, her mentorship, commitment, continuous support, expertise and above all, passion for science were instrumental in the design, development and completion of this study/thesis. Being new to this field, I am very grateful for her guidance and the time she has spent with me from discussing the design of the project to providing guidance/feedback in preparation of the thesis/manuscript writing. It has been a great honor to work under her supervision.

I would also like to thank my colleagues: Zeinab Sharifi, Areej Al Rabea, Dr. Gloria Assaker, Dr. Paul Daniel, Dr. Ahmad Chaddad and as well, Brian Meehan and Dr. Michelle Zeinieh for the exchanges of knowledge and skills, for moral support, for helping me to resolve technical issues, and for all the great memories. I am also grateful to our Administrative Research Assistant, Ms. Elena Popova, for all her help with the lab and ensuring everything was running smoothly. As well, to the Research Institute of the McGill Health Center Technical Services team for ensuring all equipment were functioning properly and to the Cancer Research Program for organizing weekly seminars to help us expand our knowledge in current cancer research.

I would like to express my appreciation to members of my thesis supervisory committee: Dr. Bertrand Jean-Claude, Dr. George Shenouda, Dr. Martine Culty and Dr. Bertrand Turcotte for their constructive feedback, to Drs. Bassam Abdulkarim and Abu S.M Norman for their input in the experimental design, and to the Division of Experimental Medicine team for their help and guidance with administrative affairs.

Finally, I cannot thank my parents, brother, and dear friends enough for all their support and motivation throughout this venture.

Preface

This thesis is presented based on the manuscript-based thesis guidelines provided by the University and contains a manuscript in preparation for submission to Scientific Reports Nature Publishing Group. Chapter 1 is a literature review of Glioblastoma Multiforme, MGMT, p53, an overview of PRIMA-1MET, redox balance and provides an introduction of the project (rationale, hypothesis, and aims). Chapter 2 reports our findings from investigating p53-independent effects of PRIMA-1MET and the role of MGMT in the differential effects of PRIMA-1MET in GBM. Lastly, chapter 3 provides a general discussion of these findings, limitations, implications and summary. This thesis is written by myself with guidance from my supervisor, Dr. Siham Sabri.

Contribution of Authors

Rehka Whyshnavy Raveendrakumar performed all in vitro studies: drug treatments and analyzed its effects via Western Blot analysis, MTT assays and ROS assays, and prepared the manuscript/thesis. Rehka Raveendrakumar is also a recipient of the Fonds de Research Masters Training Award 2017-2018.

Drs. B. Abdulkarim, B. Jean Claude and A.S.M. Noman contributed to the design of experiments, reviewed and approved the manuscript.

Dr. Siham Sabri was responsible for the conception, design, development of methodology, data analysis, manuscript/thesis revision and provided grant-funded financial support to conduct this project.

List of Abbreviations

AMPK- adenosine monophosphate-activated protein kinase

ARE- antioxidant responsive element

ARF - alternative reading frame

ASK1 - apoptosis signal-regulated kinase 1

ATR- ataxia telangiectasia related

BER- base excision repair

BTICs- brain-tumor initiating (stem) cells

CBP - CREB-binding protein

CNS - central nervous system

DISC- death-inducing signaling complex

DNA- deoxyribonucleic acid

DRAM- damage -regulated autophagy modulator

EGFR - epidermal growth factor receptor

EORTC- European Organization for Research and Treatment of Cancer

ER- endoplasmic reticulum

FADD- Fas-associated death domain

FDA- Food and Drug Administration

GBM- glioblastoma multiforme

GOF- gain of function

GPx1 - glutathione peroxidase 1

HDAC- histone deacetylases

HIF-1α - hypoxia inducible factor

JNK- c-Jun N-terminal kinase

IDH1 - isocitrate dehydrogenase 1

IR- ionizing radiation

L-BSO - L-Buthione-Sulfoximine

LOH- loss of heterozygosity

MAPK- mitogen activated protein kinase

MDM2 - mouse double minute 2 homolog

MGMT - O6-Methylguanine-DNA methyltransferase

MMR- DNA mismatch repair

MQ- methylene quinuclidinone

Mutant p53 - mutp53

NAc - N-acetylcysteine

NADPH - nicotinamide adenine dinucleotide phosphate

NF1- neurofibromin 1

NF-κB - nuclear factor kappa beta

Nrf2- nuclear factor erythroid 2-related factor

n.s. – non-significant

O4-MeG - O4-methylguanine

O4-MeT - O4-methylthymine

O6-BG - O6-Benzylguanine

OS - overall survival

PCAF- p300/CBP associated factor

PCNA- proliferating cell nuclear antigen

PDGFRA- platelet-derived growth factor

PFS- progression free survival

PI3K - phosphatidylinositol-3 kinase

PRIMA-1/ PRIMA-1MET- p53 reactivation and induction of massive apoptosis

ROS - reactive oxygen species

RT – radiotherapy

Sp1 – Specificity protein 1

TAD - transcription active regions

TMZ- Temozolomide

Trx- Thioredoxin

TrxR1 - thioredoxin reductase 1

VEGF- vascular endothelial growth factor

VEGFA - vascular endothelial growth factor

WHO - World Health Organization

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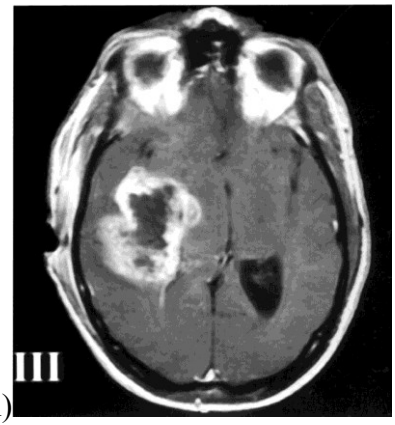
Chapter 1.
Literature Review and Introduction

1.1. Glioblastoma Multiforme (GBM)

1.1.1. GBM: Incidence, Pathology and Classifications

Glioblastoma Multiforme (GBM), currently referred to as glioblastoma is an aggressive, uniformly fatal cancer that develops from astrocytes in the brain mostly as a primary "de novo" tumor, while secondary GBM develops from lower grade gliomas (1). GBM accounts for approximately 54% of all gliomas, it is the most common and invasive primary brain and central nervous system (CNS) malignancy in adults (2). In addition, it has the highest average annual age-adjusted incidence of 3.19/100,000 population among all brain and CNS tumors and its incidence increases with age (peak incidence between ages 75-84) (1, 3, 4). A higher frequency of GBM is reported in males compared to females (3.97 vs. 2.53) with a high frequency of primary GBM being more prevalent in men (2).

GBM is diagnosed through the presence of a mass found most often in the cerebral hemispheres by magnetic resonance imaging or computerized tomography scans (5) and based on histopathological features in tissue samples, as previously defined in the World Health Organization (WHO) classification (Figure 1.1) (6). GBM consists of poorly differentiated neoplastic astrocytes and other cell types i.e. stem cells. It is morphologically and molecularly heterogeneous both within tumors and between patients (7). GBM is classified as a grade IV astrocytoma due to its rapid infiltrative growth, the presence of intravascular microthrombi necrosis with or without pseudopalisading, and/or microvascular proliferation (7). The presence of necrotic foci are a characteristic feature of GBM and can be categorized into two histological types: 1) large foci within the central area of the tumor or 2) small, irregular shaped foci surrounded by pseudopalisading cells (Figure 1.1b) (7). Pseudopalisades are a morphological feature that allows GBM to be distinguished from lower grade astrocytomas (8).

A) 

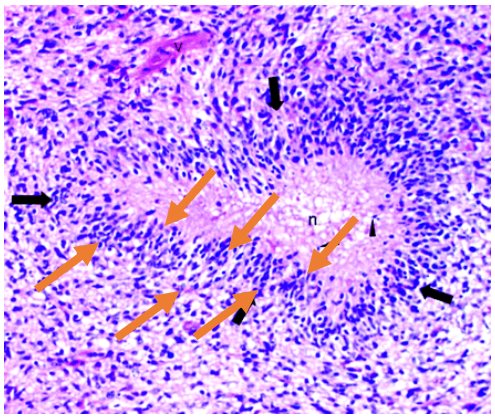
B) 

Figure 1.1. Clinico-pathology of GBM. A) MRI image of a GBM patient with high grade necrosis. Figure adapted from (9) with modifications. B) H&E stains of GBM tissue at 200X. Pseudopalisading cells (bordered with orange arrows) surrounding the necrotic foci are a morphological feature that allows GBM to be distinguished from lower grade astrocytomas. Figure adapted from (10) with modifications.

GBM most often occurs in the supratentorial regions (occipital, parietal, frontal and temporal lobes) adjacent to the ventricles in areas rich in neural stem cells and rarely in the cerebellum and spinal cord (11-14). Cerebellar GBMs, if they do occur, are more prevalent in younger patients and smaller in size compared to those in the supratentorial region (15). Apart, from being classified by its location, it can be classified into two major types based on its genetic mutations: primary and secondary GBM which are histologically indistinguishable (1).

GBM is divided into two types (primary and secondary) based on alterations of the isocitrate dehydrogenase 1 (*IDH1*) gene with approximately 90% of *IDH1 versus IDH2* mutations and R132H point mutation being the most common *IDH1* mutation (1, 7). *IDH1* mutations are identified in the initial transformation of a glial cell into a tumor cell and followed by further molecular changes corresponding to "oligodendroglial" or "astrocytic" differentiation (Figure 1.2) (1, 7). Primary GBM, is characterized by the presence of wild-type *IDH1* gene (~80-95% of cases) develops "de novo" without evidence of a precursor lesion (16). By contrast, secondary GBM (~10% of cases), which is associated with a hypermethylation phenotype, arises from lower grade gliomas with a slow progressive development (16). The mean age of patients with primary GBM is approximately 62, whereas, secondary GBM typically occurs in younger patients (mean age 45 years old) (1). Additionally, based on clinical assessment, it was found that GBM patients with mutant *IDH1* have a longer median overall survival compared to patients with a wild-type *IDH1*: (24 months vs. 9.9 months with radiotherapy (RT); 31 vs. 15 months

with RT and chemotherapy) (17). Due to the heterogeneity of GBMs, there were several other GBM classification studies conducted to stratify GBM patients into molecularly defined subgroups with the primary goal to provide better therapeutic targeted therapies. One notable work was its classification into four distinct molecular subtypes by Verhaak et al. in 2010 (18).

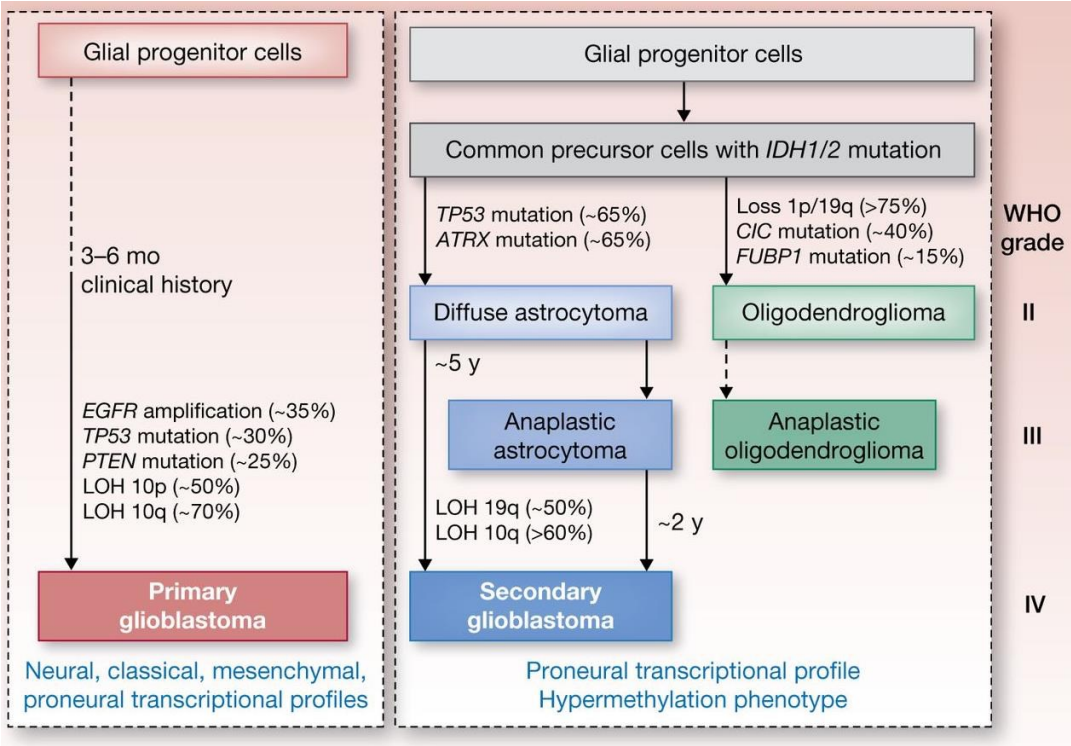


Figure 1.2. Genetic pathway for the development of primary vs. secondary GBM. Adapted from (16).

Verhaak et al. (2010) performed unsupervised hierarchical clustering of GBM data from 200 patients newly diagnosed with GBM and two normal samples to generate four distinct subtypes: proneural, neural, classical and mesenchymal (Figure 1.3) (18). The classical subtype is characterized by genomic aberrations such as epidermal growth factor receptor gene (*EGFR*) amplification, *CDKN2A* gene (encoding for p16INKA and p14ARF), *PTEN* loss and lack of *TP53* mutations (18). The mesenchymal subtype is identified by the presence of deletions and mutations of the neurofibromin 1 (*NF1*) gene, and high expression of *CHI3LI* and *MET* genes (18). The proneural subtype affects relatively younger age compared to other subtypes, displays alterations of the platelet-derived growth factor receptor A gene (*PDGFRA*), point mutations in *IDH1* and frequent mutations of the *TP53* gene (18). Lastly, the neural subtype is possible

transitory phenotype resembling normal brain tissue with overexpression of specific neuron markers (18). Among these subtypes, the classical subtype had the greatest response to standard treatment whereas the proneural subtypes was found to be the least responsive (18).

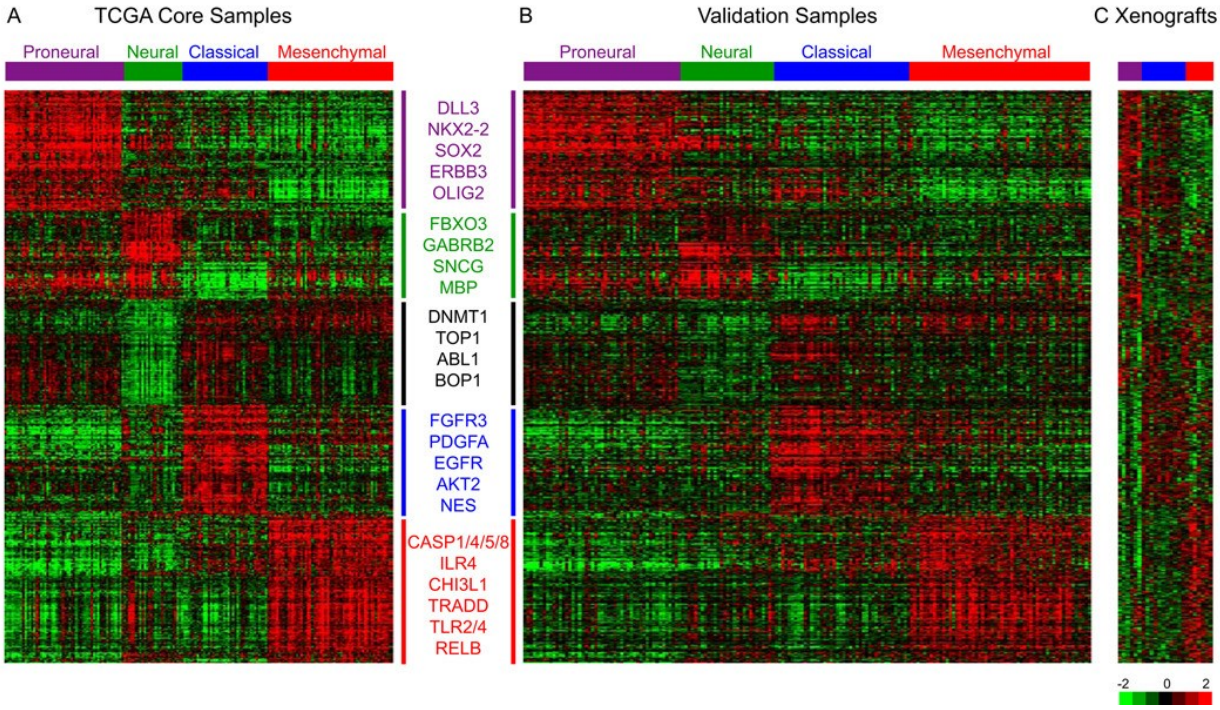


Figure 1.3. Identification of four GBM subtypes (proneural, neural, classical, and mesenchymal) from gene expression data. A) Clustering of samples based on a predictive gene list and a core set of 173 TCGA GBM samples. B) Validation of subtypes with a separate set of 260 expression profiles. C) Identification of proneural, mesenchymal and classical subtypes in 24 xenograft samples. Figure adapted from Verhaak et al (18).

Another notable GBM classification study was conducted by Sturm et al. (19) in 2012, to investigate the heterogeneity of GBM across all ages based on epigenetics, alterations of copy-number and expression patterns of both children and adult GBM cohorts. The six subgroups of GBM identified were: RTK II (Classic), IDH, K27, G34, Mesenchymal and RTKI (PDGFRA) (19). Analysis of a cohort of children with GBM (n=59) and adult patients (n=77) for DNA methylation patterns identified recurrent somatic H3F3a mutations affecting two critical amino acids of the replication-independent histone variant H3.3: K27 and G34 (19). Hence, K27 and G34 groups were associated with specific mutations of K27 and G34 residues respectively with the G34 group having genome-wide DNA hypomethylation (19). Along with K27 and G34, IDH was highly enriched in *TP53* gene mutations (19). Furthermore, the IDH group as the name

suggests have *IDH1* mutations and G-CIMP phenotype (19). All mutations in H3F3a and IDH were mutually exclusive and found to be part of different stages of development with K27 mutations found primarily in children, G34 in adolescents and IDH1 mutations in young adults (19). In addition, when they integrated DNA methylation clusters with copy number alterations, an amplification of PDGFRA was found in RTKI (PDGFRA) while the RTK II (Classic) group had a high frequency of chromosome 7 gain and chromosome 10 loss, frequent deletions of *CDKN2A*, and amplification of *EGFR* which are all hallmarks of adult GBM (19). Sturm et al. also investigated these groups in the context of previously discussed GBM types and found RTK II (Classic), RTK I (PDGFRA) and Mesenchymal groups to be in accordance with the Classical, Proneural and Mesenchymal profiles mentioned earlier, respectively (19). Surprisingly, both the K27 and IDH cluster displayed proneural expression patterns suggesting that this subgroup can be further divided into distinct groups based on methylation profile (19). The authors have also investigated the relationship between these groups and overall survival (OS) and found IDH1 mutant tumors to have a significantly longer OS than patients in K27, G34 and IDH1 wildtype groups (19). Similarly, G34 mutant patients have better OS than wildtype tumors (19).

However, despite the genetic tumor heterogeneity of GBM and its classification into distinct groups, the same multimodal treatment is given to all patients, as a result, GBM continues to remain incurable with a short survival time of 14.6 months (20).

1.1.2. Standard Treatment

Conventional GBM treatment (known as the Stupp Regimen, Figure 1.4) consists of maximum surgical resection of the tumor, followed by aggressive treatment of concurrent radiation with a chemotherapy agent, Temozolomide (TMZ) and then adjuvant chemotherapy with TMZ (20). Patients over 70 years old are given a less aggressive treatment with TMZ or RT alone (21). It is often difficult to completely resect the tumor due to GBM's high degree of invasiveness and difficulty in removing the residual tumor in some functional locations (22). Tumor cells that remain post-surgery may lead to disease progression or recurrence thus, greater safe resection of the tumor is associated with longer PFS and OS (22).

Once surgical wounds are healed, patients then begin chemotherapy and radiotherapy. Originally, postoperative RT alone was the conventional treatment for GBM until March 15th 2005, when the U.S. Food and Drug Administration (FDA) approved the use of TMZ

concomitantly with RT and alone as a maintenance therapy (20). The trial that confirmed RT with concomitant TMZ as more effective than RT alone was a phase III study conducted by the European Organization for Research and Treatment of Cancer (EORTC) in newly diagnosed patients (Figure 1.4) (20). In this study, 563 patients were randomized to receive TMZ plus RT (n=287) or RT alone (n=286) (20). Patients in TMZ+RT group received concomitant TMZ at a dose of (75mg/m^2) once daily starting from the first day of RT treatment to the last day (20). The typical dose of RT delivered was 60 Gy in fractions of 1.8-2 Gy (20). After a rest period for one month, patients were treated with six cycles of TMZ alone (150mg/m^2 or 200mg/m2; days 1-5 for every 28-day cycle) (20). The RT group received the standard RT GBM protocol of (30 fractions of 2 Gy) (20). Patients in the TMZ+RT group were found to have a significantly improved OS compared to the RT treatment alone (14.6 vs. 12 months) (20). The adverse effects for this treatment include anorexia, constipation, nausea and vomiting (20). Whole-brain RT originally used for GBM treatment was associated with various complications i.e. leukcephalopathy and neurocognitive toxicity (22). However, currently, RT is given using a three-dimensional conformal beam or intensity-modulated RT (23).

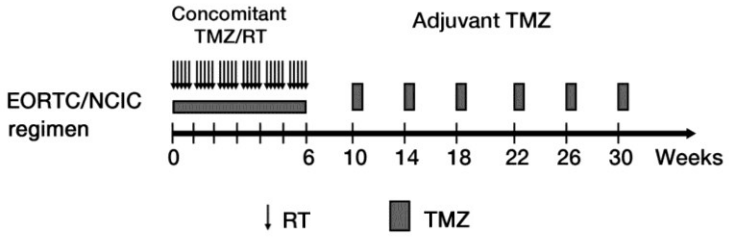


Figure 1.4. Study Schema for Stupp/EORTC Regimen GBM treatment. Figure adapted from (24) with modifications.

TMZ, is an orally administered alkylating chemotherapy drug owing to its stability under acidic conditions (25). Once it reaches its physiological pH, it reacts with water to the release both MTIC and carbon dioxide (25). MTIC reacts with water and degrades into methyldiazonium ion, a highly reactive metabolite (26, 27). The main sites of methylation for the methyldiazonium ion are the purine bases of DNA specifically at positions O6 and N7 of guanine and N3 position of adenine (28, 29). Both N3 and N7 are frequently methylated representing around 80-85% and 8-18% of the total methylations, respectively (28, 29). On the other hand, although O6 has a low methylation rate of only 5-10%, it is considered a major mediator of TMZ cytotoxicity (28, 29).

If it is not repaired, it will be paired with thymine instead of cytosine (27, 30). The DNA mismatch repair (MMR) enzyme recognizes this error and removes thymine from the daughter strand by excising only the newly synthesized strand, leaving O6-Met intact (27, 30). Thymine is likely reinserted opposite to O6-Met (27, 30) and the futile cycles of insertion and excision of thymine will ultimately lead to cell cycle arrest and apoptosis (31).

Three DNA repair pathways associated with TMZ resistance include: *(i)* DNA mismatch repair (MMR) pathway, *(ii)* base excision repair (BER) and *(iii)* O6-methylguanine DNA methyltransferase (MGMT) DNA repair protein (27) (Figure 1.5). MMR is an important mediator of TMZ cytotoxicity that repairs nucleotide base mismatches generated during DNA synthesis (32). When MGMT is not present, MMR recognizes the mismatch between O6-Met and thymine and corrects it therefore, a decrease in MMR expression corresponds to TMZ resistance (32). Furthermore, BER systems are activated by single-strand DNA breaks detected by PARP-1 (33). PARP-1 enables the recruitment of the BER complex proteins and results in repair of N3 and N7 methylations of TMZ (33). Despite low cytotoxicity of these methylations, mutations of the BER pathway contribute to TMZ cytotoxicity. Another molecule involved in resistance is MGMT which interferes (mechanism discussed below) with the cytotoxicity of TMZ. Patients with high levels of MGMT were more resistant to treatment using TMZ (34).

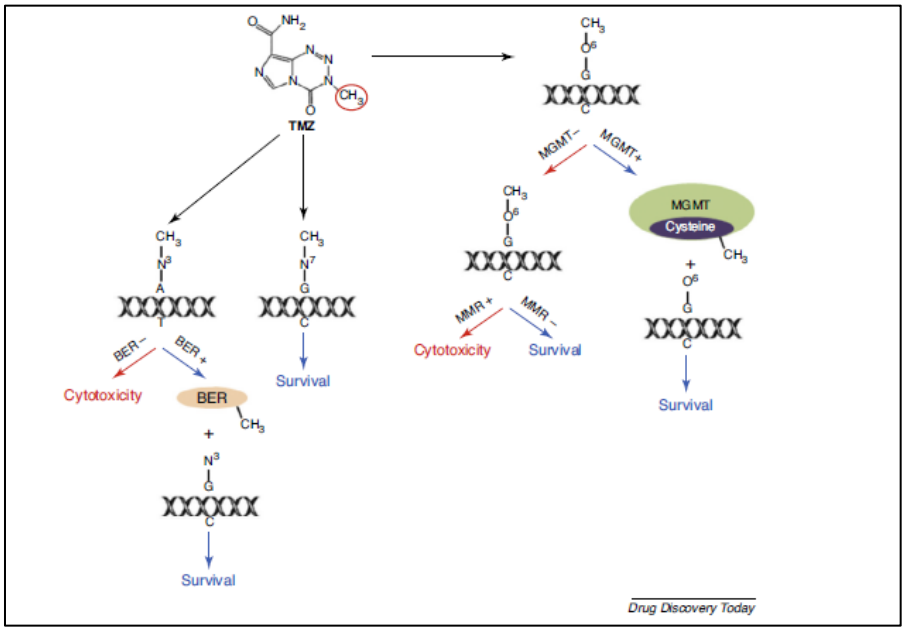


Figure 1.5. DNA repair pathways (BER, MMR and MGMT) mediate resistance to TMZ. Figure adapted from (27).

One of the major problems with GBM is that relapse post-treatment is inevitable for almost all patients. Patients newly diagnosed with GBM experience recurrence within 6-9 months' post treatment (35) and patients with recurrent GBM have a PFS of approximately 10 weeks and an OS of 30 weeks (36). This highlights the urgent need to overcome challenges in developing new therapies and treating patients. Currently there is no standard treatment offered to patients with recurrent GBM furthermore, most treatments offered provide palliative care and decrease the progression of the disease (37). For approximately 25% of patients diagnosed with recurrent GBM, surgery is recommended to improve symptoms (36). In a randomized, placebo-controlled, prospective study, it was found that patients with recurrent malignant brain tumors implanted biodegradable polymer discs with carmustine during repeat surgery had a greater 6-month survival percentage than those implanted without carmustine (38). Bevacizumab, a vascular endothelial growth factor (VEGF) inhibitor was approved in 2009 by the Food and Drug administration (FDA) to treat recurrent GBM (39). In addition, TMZ, nitrosureas using different schedules are still used to treat recurrent GBM (39). Re-irradiation with fractionated stereotactic RT (between 30-60 Gy) has also been considered and found to be beneficial for treating recurrent GBM (36). It is reported to increase OS at 6 months and 1 year (36), though with increased risk of toxicity with re-irradiation (36). GBM tumorigenesis has been correlated with the presence of a small proportion of brain-tumor initiating (stem) cells (BTICs) that are reported to be essential drivers of tumor initiation and resistant to chemo-radiation by acting as a disease reservoir (40). Besides the challenges to eradicate BTICs, GBM may shift between molecular subtypes at recurrence (41). In addition, several molecular factors have also been correlated to increased GBM recurrence i.e. mutant p53, MIB-1 labelling and O-6-methylguanine-DNA methyltransferase (MGMT) (35).

1.2. O6-Methylguanine-DNA methyltransferase (MGMT)

1.2.1. Structure

Human O6-methylguanine-DNA methyltransferase (MGMT) is a well-known small 22kDa protein that repairs O6-methylguanine (31). It is found in the long arm of chromosome 10 (10q26.3) and is transcribed into a transcript consisting of 4 introns and 5 exons spanning greater than 300kb (42, 43). MGMT has conserved structural motifs and amino acid residues involved in various DNA repair processes i.e. DNA selectivity, transfer of alkyl group, etc. (42, 43). For

example, the active site sequence consists of a proline-cysteine-histidine-arginine which is conserved in all species. The proximal promoter comprises 97 cytosine-guanine dinucleotides (CpG islands) that can be methylated (42, 43). Additionally, MGMT possesses two functional domains: C-terminal and N-terminal. The C-terminal domain consists of residues involved in DNA binding and alkyltransferase activity in the active site pocket while, the N-terminal is associated with maintaining the structure of the C-terminal domain (44, 45). These functional clusters in the active site of MGMT are shown in Figure 1.6. Thus, mutations in these regions lead to protein destabilization (42). MGMT protein displays a nuclear localization signal and is therefore, found throughout the nucleus at active sites of transcription (46).

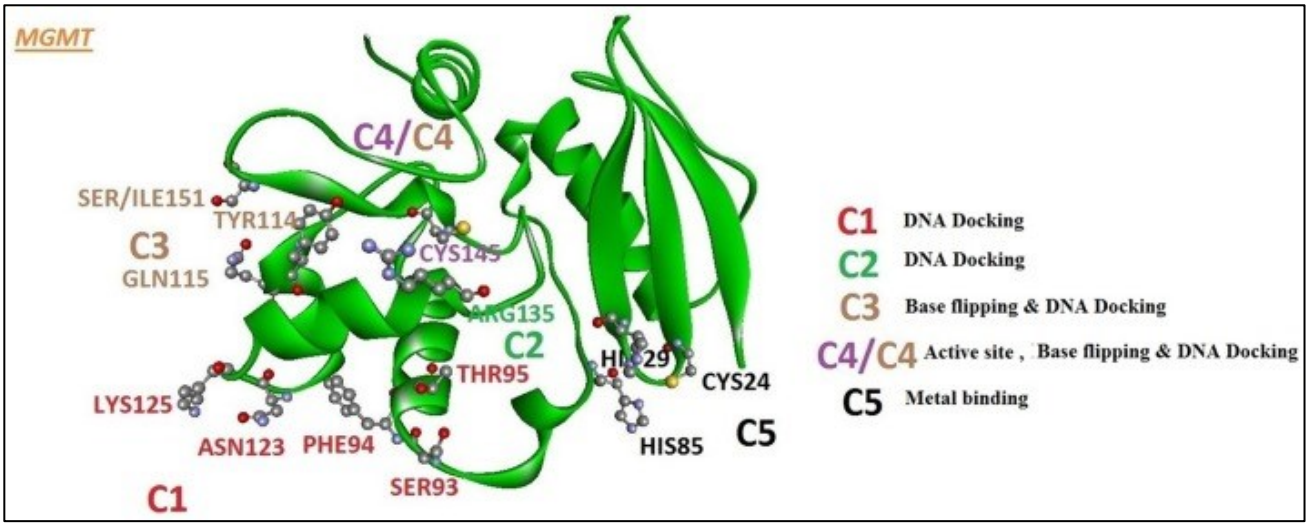


Figure 1.6. MGMT structure and its functional domain/clusters (C1-5) in the active site. The active site is involved in DNA Repair function of MGMT: DNA binding, base flipping, etc. Figure adapted from (47) with modifications.

1.2.2. Function

MGMT is the only DNA repair protein that can independently mediate its function without the involvement of a multi-enzyme complex (48). The main function of MGMT is to remove methyl groups attached to the O6 position of guanine and therefore, interfere with TMZ cytotoxicity (Figure 1.7) (49). MGMT transfers the methyl group of O6 to its cysteine residue 145 in the active site producing S-methylcysteine (49). More specifically, the transfer is accomplished following MGMT binding to the DNA and through a base-flipping mechanism which rotates the alkylated base from the DNA helix into its own active site (49). The adduct is

then covalently transferred to the cysteine residue (49). The formation of S-methylcysteine results in its deactivation and signals the protein for its degradation by the proteasome (49). Besides O6-MeG, MGMT can also remove alkyl adducts from O4-methylthymine (O4-MeT), O6-ethylguanine and O6-chloroethylguanine by transferring the methyl group to its active site (50). However, among the adducts, both O6-MeG and O4-MeT are the most carcinogenic and if left unrepaired, can lead to incorrect base insertion at a rate of 90% (50).

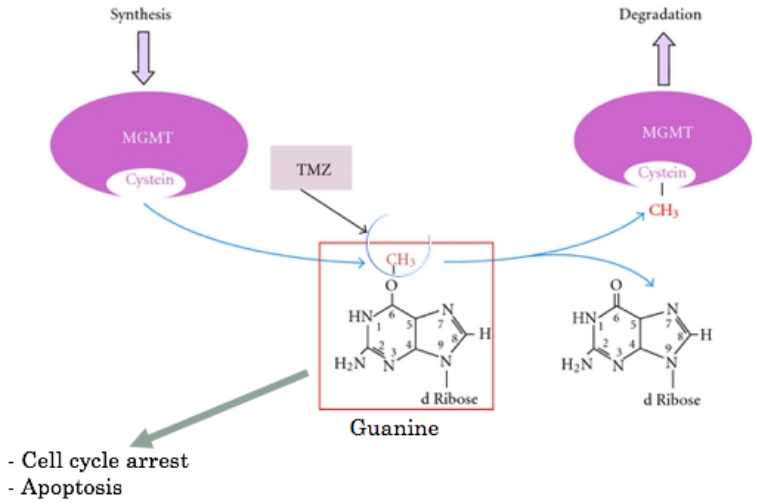


Figure 1.7. MGMT mechanism of TMZ resistance. MGMT interferes with TMZ cytotoxicity by removing the cytotoxic methyl adduct from O6 position of guanine. Figure adapted from (51) with modifications.

Due to the one-to-one stoichiometric relationship between MGMT and the number of methyl group transferred, the level of MGMT expression determines the extent to which a cell mediates resistance to alkylating agents (48). It is reported that the level of MGMT expression correlates with the methylation profile of *MGMT* promoter such that the *MGMT* gene is silenced by hypermethylation of the promoter CpG islands (52). Methylation is also associated with better outcomes for patients undergoing TMZ and RT (53). The first study to propose the role of MGMT as an important predictor of GBM treatment was by Hegi and colleagues who reported that patients having tumors with *MGMT* promoter methylation had an OS of 18.2 months whereas, patients with unmethylated *MGMT* promoter had 12.2 months OS regardless of treatment regimen (34). Based on Stupp et al.'s study (20), patients with methylated *MGMT* promoter treated with TMZ and RT were found to have an advantage based on long-term analysis of standard treatment in comparison to patients with unmethylated *MGMT* promoter

(methylated *MGMT* promoter 2 and 5 year survival: 49% and 14%; unmethylated *MGMT* promoter: 15% and 8%, respectively) (54).

Originally, MGMT was believed to have only DNA repair functions however, several studies have proposed non-repair functions of MGMT. Using MGMT-sepharose affinity chromatography and tandem mass spectrometry in colon cancer cells, it was found that MGMT interacts with over 60 proteins associated with i.e. cell cycle progression, DNA replication, translation, histones, heat shock proteins and topoisomerase I (55). Moreover, MGMT may act as an ancillary protein responsible for other functions by interacting with different binding partners (55). For example, the transcription complex, CREB-binding protein, CBP/p300 is involved in the co-activation of various transcription factors (56). Interestingly, MGMT was found to co-precipitate with this complex and to be constitutively present at the active transcription sites, suggesting a role of MGMT in activating various genes (56). In addition, it was found to physically interact with human proliferating cell nuclear antigen (PCNA), a homotrimeric DNA clamp important for replication, through reciprocal co-immunoprecipitation (56). In a recent study from our lab, Chahal et al. reported a direct link between MGMT expression and decreased angiogenesis (57). MGMT was associated with differential levels of a vascular endothelial growth factor receptor 1 and 2 (VEGFR-1 and -2) and vascular endothelial growth factor A (VEGFA) such that MGMT-positive cells have an antiangiogenic profile with high VEGFR-1/VEGFA ratio (57). Taken together, these studies suggest non-repair functions of MGMT that could be also involved in GBM resistance to standard treatment and response to other therapies.

1.2.3. MGMT regulation

MGMT is variable across different stages of development, cell/cancer types, cell cycle phases, among individuals, and across tissues with the highest expression found in the liver and the lowest in the brain (58). It also frequently found in hypoxic central cores (59). There are various ways and stages by which MGMT is regulated whether it is at the gene level, post-transcription or after translation. *MGMT* promoter methylation represents an important mechanism of regulation (60). Methylation of CpG sites in the promoter prevents binding of transcription factors to the promoter preventing MGMT protein expression (60). This silencing effect is highly dependent on the extent by which the MGMT promoter and its residues are methylated, as certain CpG dinulcleotides are more effective in silencing the gene than others

(61). For instance, methylation of certain CpG sites in MGMT can affect binding of Sp1, a transcription activator (62).

Besides methylation, another important epigenetic mechanism associated with MGMT regulation is histone acetylation. Cells with high MGMT expression have acetylation of the lysine residues on histones H3 and H4 to activate its expression (63). Furthermore, under physiological conditions, phosphorylation was also reported to regulate MGMT activity and reducing activity of recombinant MGMT by 30-65% (62). In addition, phosphorylation of the nuclear localization signal in MGMT increased its nuclear localization (64).

Several transcription factors were found to activate the transcription of the *MGMT* gene i.e. AP-1, and NF-kb (62). In addition, other nuclear factors were identified i.e. AP-2, NF-IL6 and ER-alpha through *in silico* analysis (60). Other proteins have been implicated in MGMT regulation. In HCT116 cells, it was found that expression of wild-type *TP53* inhibited MGMT expression by sequestering Sp1 (65). This inhibition was reversed by overexpression of Sp1 (65). Additionally, as mentioned earlier, MGMT expression was frequently found in the hypovascular central core of the tumor (59). This may be explained by activation of the hypoxia inducible factor (HIF)-1α, which promotes MGMT expression (59).

The role of microRNAs in MGMT regulation has been investigated (66). Several interaction sites in the 3'-UTR regions of the M*GMT* gene were discovered for miRNAs (62, 66). MiRNA-767-3p, miRNA-221, -222 were found to be regulators of *MGMT* gene expression and miRNA-648 in regulating MGMT protein expression (62, 67). Genome-wide microarray-based analysis was used to identify three novel miRNAs in regulating MGMT: miR-127-3p, miRNA-409-3p and miRNA-124-3p (68). However, these miRNAs were not as effective as miR-181-5p in suppressing MGMT-mediated effect suggesting that miRNA-181d-5p is the main miRNA regulating expression of MGMT (68).

Thus, there are several mechanisms by which MGMT is regulated. It is important to understand these mechanisms to be able to develop therapies targeting this protein.

1.2.3.1. p53 and MGMT

Another molecule that regulates MGMT is the tumor suppressor protein, p53. Induction of both exogenous or endogenous p53 expression in various cancer models including GBM, resulted in a significant decrease of MGMT at various stages (mRNA and protein levels) (69).

As mentioned above a possible mechanism proposed for its downregulation includes p53-mediated sequestration of Sp1, a transcription activator of MGMT (65). In contrast, there were studies suggesting opposite effects such that knockdown of wtp53 by RNAi led to a downregulation of MGMT expression in both human astrocytic glioma cells and in neonatal murine astrocytes (70). Furthermore, an interaction between mutp53 and MGMT has also been reported. Bobustuc et al. found inhibition of MGMT transcription in GBM cell lines by mutp53 (70). In addition, recently in our lab we found that silencing MGMT decreased expression of mutp53 in GBM cell lines, which suggested a reciprocal relationship between mutp53 and MGMT (71). Together, these findings suggest a complex relationship between p53 and MGMT, which needs to be specifically investigated in GBM.

1.3. Tumor Suppressor p53

Besides MGMT, the tumor suppressor protein, p53 which is associated with chemoradiation resistance in GBM (72), and will be presented in the following section.

1.3.1 Structure

In 1979, p53 protein, a tumor suppressor, was first identified in a physical complex with SV40 large T-antigen virus as a non-viral protein with a molecular mass of 53kDa (73, 74). Human p53 protein is located on the short arm of chromosome 17 (17p13) encoded by a 20kb *TP53* gene (75). It shares the same domain structure as its older homologues p63 and p73 (76), consisting of three functional domains: N-terminus, a central core and a C-terminal region (Figure 1.8) (77, 78).

The N-terminal domain (residues 1-~100) comprises a transactivation region with two contiguous transcription active regions (TAD1/2) and an adjacent proline-rich region (77). The transactivation region is involved in the interaction with other proteins, whereas the proline-rich region is involved in apoptosis and in maintaining p53 stability (77). The N-terminal region is believed to be phosphorylated mostly within the nucleus by various protein kinases i.e. c-Jun N-terminal kinase, DNA-PK, cyclin-dependent activating kinase, ataxia-telangiectasia mutated kinase and ataxia-telangiectasia related kinase (79). It is also involved in interacting with the C-terminus to regulate certain p53 functions (80). Furthermore, it was found that the N-terminus

contains an auto-inhibitory region that is mechanistically different from the C-terminus and that controls the dissociation of p53 from the p53-binding site (77).

The highly conserved central core DNA binding domain (residues ~100-300) is involved in targeting specific consensus sequences in the promoters of p53-regulated genes (77). This domain also manifests 3'-5' exonuclease activity, and interacts with the internal regions of single-stranded DNA. Point mutations in this domain are often seen in different cancer types because it plays an important role in mediating p53 functions (77).

Lastly, the C-terminal domain (residues 300-400) consists of a flexible linker, a tetramerization (oligomerization) domain and basic C-terminal DNA domain (77). The tetramerization region contains a negative regulatory domain that affects p53 function by keeping the protein in an inactive state for sequence-specific DNA binding (81). However, this effect can be overcome through phosphorylation by kinases i.e. protein kinase C, which stimulates p53 to bind DNA sequence-specifically with high affinity (81). Furthermore, deletion of the C-terminus is associated with sequence-specific DNA binding of p53 (81). It also consists of sequences important for nuclear localization (82) and is essential for p53 role in DNA repair and recombination i.e. DNA strand re-association, binding to strands with insertion/deletion mismatches, or DNA damaged chemically/IR (78).

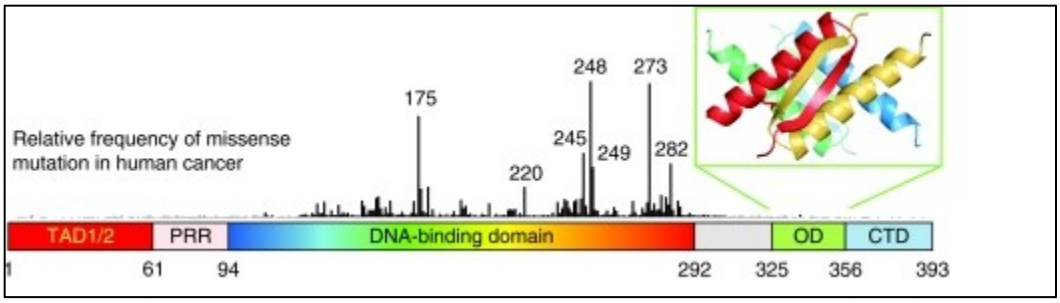


Figure 1.8. Structure of p53. p53 consists of three functional domains: N-terminal domain (two transcriptional active regions (TAD1/2) and adjacent proline-rich region (PRR)), the DNA binding domain and the C-terminal (the flexible linker (grey region), tetramerization domain (OD), and basic C-terminal DNA domain (CTD)). Figure adapted from (83) with modifications.

1.3.2. Regulation of p53

One key regulator of wtp53 levels is the Mouse double minute 2 homolog (MDM2) protein, an E3 ubiquitin-protein ligase encoded by the *MDM2* gene with its human homologue being, HDM2 (84). It mediates its regulatory effects via two mechanisms: first, it binds to the

highly-conserved transactivation domain of p53 in the N-terminus and sterically inhibits transcription by interfering with the recruitment of basal transcription machinery components (85). This is further validated in a mutational analysis study of p53 N-terminus where residues required for p53-mediated transcriptional-activation overlapped significantly with residues required for interacting with MDM2 (86). The second mechanism is that when wtp53 activity is not required for cell survival, it promotes p53 protein degradation by promoting its ubiquitination and subsequent proteasomal degradation (87, 88).

p53 also stimulates transcription of the *MDM2* gene by binding to its promoter (87, 88). The promoter of the *MDM2* gene contains a p53-binding site and is transcribed in a p53-dependent manner (87, 88), suggesting the presence of a negative feedback regulatory loop for p53 levels wherein once p53 is activated in response to DNA damage, it leads to up-regulation of the MDM2 protein to maintain p53 levels low under normal physiological conditions (87, 88). This serves to maintain p53 at low levels and to terminate p53 signal once stress signals are gone. p53 stimulation of MDM2 transcription occurs later than other p53-target genes thus, limiting the time for p53 to exert its biological and biochemical effects (87, 88). When MDM2 protein level is not present, p53 becomes deregulated and exceeds activity levels eventually leading to embryonic death (89). MDM2-knockout mice showed early embryonal lethality that was reversed by simultaneous inactivation of p53 (89). On the other hand, increased expression of MDM2 is associated with inhibition of p53 and as a result leads to cancer (87). MDM2 is also described to regulate p53 by modulating its stability. Normally, in healthy cells, MDM2 translocates p53 from the nucleus into the cytoplasm, where p53 is targeted for ubiquitin-dependent proteasome-mediated degradation (90). However, under stressful conditions i.e. DNA damage, increased levels of p53 in the nucleus result in the disruption of MDM2 ability to target p53 for degradation (90).

Furthermore, there are two mechanisms proposed to explain the disruption of p53/MDM2 function. The first is through an alternative reading frame (ARF) in the locus encoding the CDK inhibitor p16 which can prevent MDM2-mediated degradation by binding MDM2 (91). However, ARF is not essential for p53 activation in response to stress as it was shown that ARF-/- cells are still capable of inducing p53 (91). The second mechanism by which p53/MDM2 interaction is disrupted is through phosphorylation of serine-15 and/or serine-20 in response to DNA damage which leads to reduced interaction between p53 and MDM2 (92). Another

important regulator of p53 is MDMX, a member of the MDM2-family. MDMX dimerizes with MDM2, facilitating E3 ligase activity which contributes to p53 degradation (93). Other regulators of p53 are microRNAs such as miRNA-125a and miRNA-125b which downregulate p53 and suppress apoptosis (94).

Once p53 is activated, it undergoes various forms of post-translational modifications of which phosphorylation and acetylation are two major types that modulate p53 activity (95, 96). In vitro, p53 is phosphorylated on the N-terminal and C-terminal regulatory domains by various kinases i.e. cyclin dependent kinases, casein kinase I/II, protein kinase C, mitogen-activated protein kinase, etc. (96). The residue that is phosphorylated determines the interaction between p53 and other proteins (96). For instance, phosphorylation of the N-terminus is associated with inhibition of the interaction with MDM2, whereas phosphorylation of the C-terminal sites triggers sequence-specific DNA-binding potential of the protein (95, 96).

The other post-translational modification known to modulate p53 activity is acetylation (97). Acetylation is important for its efficient recruitment of cofactors and for activation of its target genes in vivo (98). Specifically, a transcriptional co-activator of p53 which acetylates lysine K320 of p53 is p300/CBP associated factor (PCAF) (99). Through this, it can either promote MDM2-mediated ubiquitination of p53 and its degradation or acetylate lysine residues of the C-terminus of p53 such that it maintains its stability or acetylate the histones (100, 101). Other acetyltransferases of p53 are histone acetyl-transferase enzymes, Tip60 and hMof are cofactors recruited by p53 (102). K120, a conserved residue in the DNA-binding core domain, is acetylated by hMOF and TIP60 acetyltransferases and is important for p53-mediated activation of the apoptotic proteins, PUMA and BAX. (102). Acetylation increases in response to stress leading to p53 activation and stabilization (103). Furthermore, several studies have proposed that C-terminal acetylation has some cell type-specific regulatory effects (102). For instance, loss of acetylation at certain C-terminal lysines completely abolishes the ability of p53 to activate p21 and suppress cell cycle progression (102). Furthermore, deacetylation i.e. histone deacetylases (HDACs) complexes were found to repress the ability to activate p53, induce apoptosis/ growth arrest by allowing MDM2-mediated p53 degradation (104).

1.3.3. Functions of p53

1.3.3.1. Overview

p53, a potent tumor suppressor and transcription factor, that is activated in response to stress, such as oncogene expression, hypoxia, ribosomal dysfunction, DNA damage, etc. (Figure 1.9) (105). This protein then, leads to the activation of various mechanisms i.e. cell cycle arrest, DNA repair mechanisms, regulation of oxidative stress, angiogenesis, cell survival, modulation of autophagy, apoptosis or senescence (Figure 1.8) (75, 105). Therefore, these functions of p53 explain why cells lacking p53 or harboring *TP53* mutations promote tumor progression (106). Moreover, some TP53 missense mutations may confer novel oncogenic properties described as mutant (mut)p53 "gain-of-function' (GOF), and were associated with increased tumor invasion, metastasis in lung cancer and chemoresistance (106).

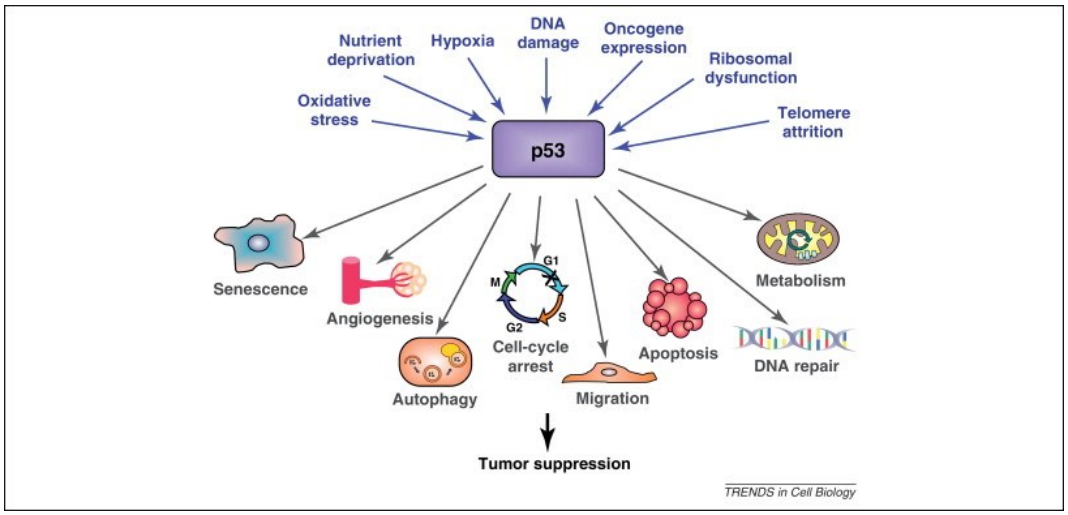


Figure 1.9. Activation of p53 and its role in tumor suppression. Figure adapted from (107).

1.3.3.2. Activation and functions of wildtype p53 (wtp53)

The process of wtp53 activation can be described in three steps: 1) stabilization of p53 and prevention of its degradation, 2) sequence- specific DNA binding and 3) transcriptional activation of its target genes (108). An important protein that determines stability of p53 is MDM2, described above (109). Upon genotoxic stress, i.e. DNA damage, p53 is post-transcriptionally modified by various kinases i.e. ATM, ATR, DNA-PK, Chk1 and Chk2 that phosphorylate specific sites in the N-terminus of p53 (97). Phosphorylation of sites in the N-terminus prevents MDM2 from binding and therefore, results in p53 stabilization (97). The interaction between p53 and MDM2 can be further inhibited by a tumor suppressor p14ARF in response to Ras or Myc oncogene expression (110).

Once p53 is stabilized, it then binds to its target DNA in a sequence-specific manner and as a tetramer through the control of tetramerization domain (111) and activated targets depend on cell type, stress, tumor microenvironment, etc. (78). p53 acts as a transcription factor that both activates or represses its target genes by interacting with other factors i.e. TFIID/TAFs (101). Post-translational modifications of p53 mentioned earlier can also influence the interaction of p53 with other transcriptional activators and repressors/corepressors (101).

In response to stress, three different classes of p53-targets are activated (112). First, activation of genes that protect cells from excess of p53 activation (i.e. Mdm2), which otherwise would be detrimental for cell survival (75, 112). The second class of p53-target genes induces growth arrest and initiates DNA repair (i.e. p21) (75, 112). The third class of target genes induces an irreversible apoptotic response i.e. PUMA, BAX, NOXA, etc. (75, 112). p53 is therefore, important in many different pathways crucial for its tumor suppressor activity (75). For example, in cell division, to ensure that each process of cell cycle is accurately completed , there are checkpoints in place that force damaged cells to undergo growth arrest and to prevent propagation of DNA mutations to daughter cells (75). p53 also induces G1 arrest through transactivation of p21 Waf1/Cip1, a cyclin-dependent kinase inhibitor and perturbs the function of cyclin B1/cdc2 complex to promote mitosis from G2 phase (75). Hence, through these mechanisms p53 is associated with cellular senescence, a permanent form of cell cycle arrest induced by dysfunctional telomeres, disruption of chromatin organization, etc. (75). As mentioned earlier, p53 is also associated with autophagy, a process that leads to the destruction of the cell in response to injury using a lysosomal machinery and which allows recycling of destroyed cell organelles for new cells (75). It was previously reported that p53 induces transcriptional activation of the damage-regulated autophagy modulator (*DRAM*) gene which encodes for a lysosomal protein involved in autophagy, along with other autophagy promoting genes *Isg20L1*, and *Atg7* (75).

Lastly, with respect to apoptosis, a tightly controlled cell-death process, p53 promotes apoptosis by both transcription-dependent and independent mechanisms. First, p53 transactivates various pro-apoptotic genes such as the Bcl-2 family genes (i.e. Bax, PUMA (p53-up-regulated modulator of apoptosis), Noxa, etc.), PIG3, CD95 (Fas), etc. in a transcription-dependent manner (113). Each of these genes possess consensus p53-response elements in their promoters (114). In addition, p53 can transactivate components of the apoptotic effector machinery i.e. the gene

encoding Apaf-1, a co-activator of caspase-9, and upregulate caspase-6, an effector caspase (115). For the extrinsic pathway, p53 is also known to activate genes i.e. Fas/CD95 and DR5, the ligands of cell death receptors (115). It can also translocate to the mitochondria where it induces the mitochondrial outer membrane permeabilization protein to release pro-apoptotic factors from the intermembrane space (113). Furthermore, PUMA, a key mediator of both p53-dependent and independent apoptosis is activated by p53 in response to genotoxic stress, it translocates to the mitochondria where it binds to Bcl-2 family proteins (Bcl-XL) to release p53 and activate Bax (113). Furthermore, both Bax and PUMA can induce release of ROS from the mitochondria that lead to p53 activation to repress both NOS2 and upregulate GPx to decrease ROS levels. p53 is therefore involved in regulating redox balance as it is a redox sensitive protein (116). By contrast, it can also mediate apoptosis through a ROS-dependent pathway by stabilizing or activating p53-regulated apoptotic proteins (116). p53 also represses genes such as survivin coding for an IAP capable of inhibiting apoptosis when overexpressed (117). Thus, activation of apoptosis by p53 is extremely important such that disruption of this process promotes continued proliferation of damaged cells, genomic instability, anti-apoptosis, and ultimately leading to tumor progression (115).

1.3.4. Mutant p53 (mutp53)

More than half of all cancers exhibit alterations in *TP53* gene, which either attenuate or eliminate its tumor suppressor function (72). Mutant p53 (mutp53) like its wildtype counterpart is activated in response to stress to mediate its respective functions whether it is the role of wtp53 in tumor suppression or mutp53 through its gain-of-function (GOF) properties and/or dominant negative effect on wtp53 in cancer promotion (72). Furthermore, compared to wtp53 which returns to low basal levels after stress, mutp53 remains stable and is found at high levels of expression in cells (118). This can be explained by the lack of the negative feedback loop in mutp53 and other negative regulators of p53 (118). Thus, in cancer cells, mutp53 levels are elevated following chronic stress and its accumulation is crucial in the development of oncogenic GOFs activities (119). p53 mutation is therefore, a key event in tumorigenesis leading to deregulated cell cycle, genomic instability i.e. gene amplification, enhanced cell growth and DNA synthesis, resistance to stress signals i.e. chemotherapies, promotion of angiogenesis, metastasis and invasion (120).

1.3.4.1. Mutant p53: missense mutations and dominant-negative effect

Alterations of the *TP53* gene can occur via two genetic mechanisms: 1) deletion of the *TP53* gene and 2) mutations that attenuate p53 function (87). One-third of *TP53* alterations are reported to result from haploinsufficiency of the tumor suppressor gene (72). Unlike most tumor suppressor genes where the primary mutations are nonsense or deletions, many cancer-associated mutations in *TP53* are missense mutations (72). This type of mutation represents 75% of all p53-mutant forms in humans and are generally classified into two different classes: conformational or DNA-contact (72). The first class, DNA-contact are missense mutations in the DNA-targeting sequence whereas the second class, conformational are missense mutations that disrupt p53 structure (121). Conformational mutations can result in a conformation of p53 protein which displays loss of the DNA-binding activity (121). In the context of the *TP53* gene, most of these mutations are found within the DNA-binding domain, resulting in the production of an altered p53 (121). The frequency of mutations in the DNA-binding domain of *TP53* is dependent on various factors such as the type of cancer and the tumor microenvironment (122). The most frequent mutations in this gene are known as "hot spot" residues and they include R175, G245, R248, R249, R273 and R282 (Figure 1.8) (123). These highly frequent mutations account for approximately one-third of the *TP53* missense mutations and are believed to arise from spontaneous deamination of methylated CpG dinucleotides (123). However, other CpG dinucleotides that are not mutated are also present in p53 suggesting that these mutations may be important for inactivating wtp53 or they promote distinct activities for the protein (123). It is also important to note that not all missense p53 mutants are equal such that some exert GOF effects, while others may only exert a dominant negative effect on wtp53 activity (72).

A unique feature of some of these missense mutations is that they result in the production of stable p53-mutants that can exert a dominant negative effect on the wild-type allele therefore, disrupting its function (124). Specifically, missense mutations in humans are followed by loss of heterozygosity (LOH) at the corresponding locus which may explain the "gain of function" hypothesis (120). Among the *TP53* missense mutations in cancer, 60% show LOH, while 40% do not (120). For missense mutations that do not undergo LOH, wtp53 expression and function is suppressed by forming complexes that suppress activity through conformation shifts or by inhibiting the DNA-binding domain ability of wtp53 on target genes (124). Furthermore, mutp53

was found to exert GOF activity that is independent of the dominant-negative effect (124). GOF is mediated by two main components: 1) stabilization of mutant p53 and 2) interactions with mutant p53-interacting partners (120) and a few examples of GOF properties include resistance to treatment, enhanced cell proliferation, and prevention of apoptosis. The primary mechanism of mutant p53/GOF involves interacting with transcription factors or chromatin-modifying proteins that alter gene expression (125, 126).

1.3.4.2. Mutant p53 and its interacting partners

It is also important to consider the differences between the different types of mutations of p53 as it may determine which mutant p53-binding partners it will bind to with high affinity and the genes it targets (120). The type of mutp53-interacting proteins and their presence in the nucleus where most interactions occur is dependent on various factors i.e. type of tissue, pH, inflammation, etc. (120). The transactivation domains of p53 are critical for the pro-oncogenic functions of p53 mutants (72). Mutp53 are reported to transactivate genes involved in tumorigenesis, for example, MYC, PCNA, CDK1, CDC25, and EGFR. It can also upregulate genes involved in inhibition of apoptosis or promotion of chemoresistance i.e. EGFR1, IGF2, NFKB2, TIMM50 (127, 128) and upregulate genes involved in indefinite replication i.e. TERT and cooperate with NRF2, a transcription factor associated with increased degradation for tumor suppressor proteins and increased resistance to proteasome inhibitor (72). It was also found to increase expression of various target genes normally repressed by wtp53 such as Sp1, and NF-kB (129). GOF properties may be further mediated through the formation of protein complexes with several partners. Furthermore, mutp53 can also modify chromatin architecture by binding and activating chromatin modifying enzymes (130).

However, amongst these mutp53-binding partners, the most studied interacting partners are p63 and p73, members of the p53 family (76). Both p63 and p73 can form homotetramers and heterotetramers with each other, whereas wtp53 is unable to form a heterotetramers with either p73 or p63 (131). It is reported that certain tumor-derived mutants of p53 can promote chemoresistance, migration, invasion and metastasis by binding to p63 and/or p73 and inhibiting its function (132). This is supported by studies from Gaiddon and Strano where they found that the mutated core domain in mutant p53 is sufficient for binding to p63 and p73 (132).

1.3.4.3. Regulation of mutp53

Most regulatory pathways between mutp53 and wtp53 are relatively the same (106). However, mutp53 does not have a negative feedback loop as seen with wtp53 because it is believed that mutp53 prevents the transactivation of MDM2 by p53 (133). Moreover, mutp53 can be degraded by alternative forms of autophagy i.e. macroautophagy or through interaction with heat shock proteins that target p53 to the lysosomes (134). It is protected from CHIP-mediated ubiquitination and MDM2-mediated degradation through chaperone proteins by forming a complex with the chaperone protein, Hsp90 (135). In addition, ribosomal proteins were found to regulate MDM2-p53 interaction and activate wtp53 i.e. RPS27 that is repressed by wtp53 and stabilizes mutp53 (136). Acetylation of mutp53 is involved in its stabilization (137).

MiRNAs play an important role in regulating wtp53 and mutp53 (138). Specifically, with p53, miRNAs cannot discriminate between its wildtype and mutant forms of mRNAs unless it is targeting a mutated site, thus, microRNAs can regulate both expression of wtp53 and mutp53 (138). Mutant p53 can also be regulated by miRNAs by targeting the mRNAs of negative regulators of p53 (139).

In sum, it is very important to understand the functions, interacting proteins and regulation of mutp53 to be able to effectively target mutp53 in various cancers.

1.3.5. p53 and GBM tumorigenesis

TP53 missense mutations as mentioned above may confer novel oncogenic properties, previously described as mutp53 GOF that promote tumorigenesis. Alterations of the *TP53* gene is reported to be important for both primary (30%) and secondary GBM (60%) tumorigenesis (1). Furthermore, in the context of GBM, the relationship between p53 and MGMT is being extensively studied. Previous studies have suggested the role of wtp53 in the negative regulation of MGMT levels (65) and recently in our lab, we showed that MGMT silencing led to a decrease in mutp53 (71). These findings suggest that rescuing wtp53 function may concomitantly lead to decreased levels of MGMT in GBM and therefore, increase sensitivity to alkylating agents such as TMZ (71).

1.3.6. Targeting mutp53 for cancer therapy

Small molecules that can either reactivate mutp53 to a wtp53 conformation or destabilize

mutp53 were proposed as effective ways to circumvent mutp53-mediated tumorigenesis. These strategies have been extensively studied in the last two decades with nutlins being the first molecules discovered to prevent MDM2 (140). In addition, through cell-based screening for other inhibitors of p53-MDM2 complex, i.e. RITA, "reactivation of p53 and induction of tumor cell apoptosis" was identified and found effective in inducing p53-dependent or independent cell death (141). Several approaches were proposed to reactivate mutp53 such as the use of short peptides that either activate the DNA-binding properties of certain p53 mutant isoforms or that act as chaperones to stabilize wtp53 (142). However, among all the different compounds identified to target mutp53, the most promising compounds are PRIMA-1 and its structural analog, PRIMA-1MET (APR-246), (143) which has been studied in various cancer models, alone or in combination with other therapies and has also shown compelling results both in vitro, in vivo and in clinical trials (144).

1.4. PRIMA-1MET

1.4.1 Overview

PRIMA-1 (chemical name: 2,2-bis(hydroxymethyl)-1-azabicyclo[2,2,2]octan-3-one) an abbreviation for "p53 reactivation and induction of massive apoptosis" was first identified by Bykov and his colleagues in 2002 (143). This molecule along, with its structural analog, PRIMA-1MET (APR-246) was discovered through a cell-based assay to identify compounds that can restore wild-type function to mutp53 of 2000 low-molecular weight compounds from the National Cancer Institute library (143). PRIMA-1MET is a methylated and active derivative of PRIMA-1, possessing increased permeability properties (144). It is the first of its class to show both safety and a favorable pharmacokinetic profile, which enabled its testing in a phase II clinical trial for patients with recurrent mutp53 high-grade serous ovarian cancer (NCT02098343) (71). Moreover, the first-in-human study (NCT00900614) was in hematologic malignancies and prostate cancer where it was well-tolerated, displayed favorable pharmacokinetic profile, and increased apoptosis through the upregulation of p53-target genes (145). Several pre-clinical studies have been conducted to understand its underlying mechanism of action. Although PRIMA-1 and PRIMA-1MET were believed to only to inhibit and induce apoptosis in cancer cells carrying mutp53, they also exhibited cytotoxic effects irrespective of p53 status (144). In fact, p53-independent effects of PRIMA-1MET were observed in many

different cancers suggesting that alternative mechanisms may be in place.

1.4.2. Mechanism of Action

PRIMA-1 decomposed to the active metabolite, methylene quinuclidinone (MQ) (Figure 1.10) (146) which Lambert et al. reported to be a Michael acceptor, that reacts covalently with thiol groups of both wtp53 and mutp53 (146). More specifically, it reacts with the Cys124 residue located in the center of the pocket between loop L1 and sheet S3 of the p53 core domain (147). Other possible cysteine residues include Cys182, Cys229, Cys242 and Cys277 which are all exposed on the surface of the p53 core domain (148). Both PRIMA-1 and its analog, PRIMA-1MET function in a similar manner. PRIMA-1MET was shown to reactivate mutp53 by physically interacting with p53 resulting in the refolding of the p53 core domain to its wtp53 form and restoring transcription activity of p53 (149). Furthermore, as mentioned above, it was found to be effective in cells irrespective of p53 status and mutation type (150).

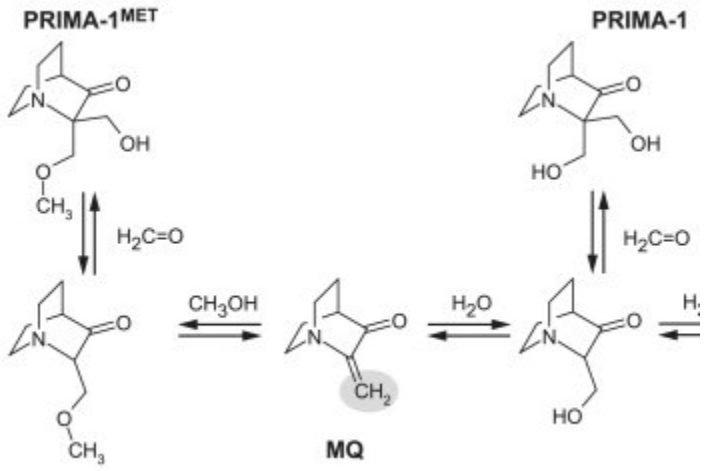


Figure 1.10. Decomposition of PRIMA-1 and PRIMA-1MET to its active metabolite, MQ, a Michael acceptor. Structures of PRIMA-1MET, PRIMA-1, and MQ. The CH2 group (shown in grey) is the most important reactive group for MQ. Figure adapted from (146).

Three p53-independent mechanisms were proposed: 1) reactivation of inactivated/unfolded wtp53, 2) induction of oxidative stress and 3) activation of the unfolded protein response (UPR) (Figure 1.11) (144). First, it is reported that MQ can bind to and activate unfolded wtp53 therefore, increasing levels of wtp53 (151). Second, PRIMA-1MET is shown to induce intracellular ROS in various cancer types such as epithelial ovarian cancer and acute myeloid leukemia (151-153). The importance of ROS induction in the cytotoxicity of PRIMA-

1MET is supported by the fact that the ROS scavenger, N-acetyl cysteine (NAC) antagonizes with PRIMA-1MET, inhibiting its effect on cell proliferation and apoptosis (153). Additionally, MQ was found to bind to both glutathione (GSH), an antioxidant that regulates levels of ROS, and free intracellular cysteines required for glutathione synthesis in both myeloma and ovarian cancer cells (153, 154). PRIMA-1MET was reported to synergize with buthionine sulfoximine (BSO), an irreversible inhibitor of glutathione synthesis and to induce cell death irrespective of p53 status (153, 154).

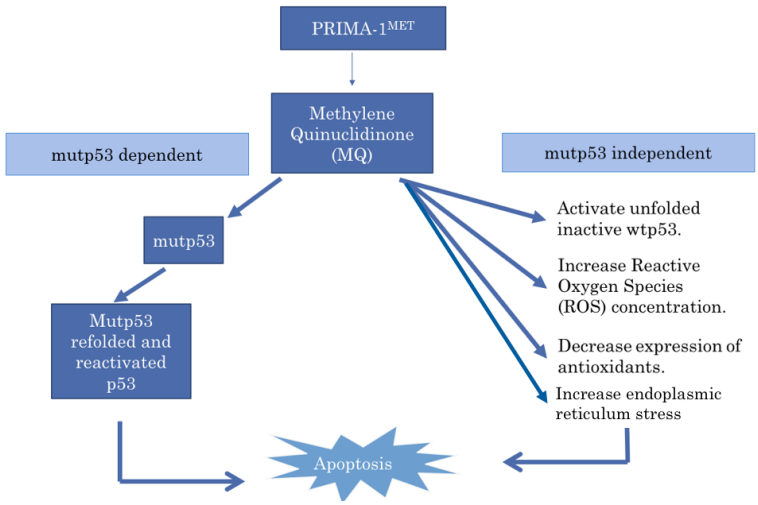


Figure 1.11. PRIMA-1MET-mediated p53-dependent and independent mechanisms. (144, 151)

In addition, other antioxidant enzymes which were reported to be inhibited by PRIMA-1MET includes thioredoxin reductase 1 (TrxR1), Prx3, or GPx-1 (144). More specifically, TrxR1, is an selenoprotein enzyme involved in the regulation of redox balance. In Peng et al.'s study, TrxR1 expression and activity was found to be inhibited irrespective of p53 status by MQ in lung adenocarcinoma and osteosarcoma cells carrying exogenous mutp53 or parental p53-null cell lines (155). TrxR1 was inhibited through the binding of MQ to the selenocysteine containing C-terminal active site motif (155). The importance of the Sec residue was shown in this study when cells with a sec-to-cys mutant of TrxR1 were not inhibited by PRIMA-1MET (155). Moreover, knockdown of the TrxR1 attenuated effects induced by APR-246, which revealed the importance of MQ binding to TrxR1 in its action (155). Inhibition of antioxidants also led to an increase in ROS levels (156).

PRIMA-1MET also activates the endoplasmic reticulum (ER) stress pathway specifically the unfolded protein response (UPR) (157). UPR is a stress response activated in response to accumulation of both unfolded and misfolded proteins in the ER to increase levels of chaperones that degrade misfolded proteins. Furthermore, if the stress is not attenuated, it can lead to cell apoptosis (144). In fact, PRIMA-1MET results in the upregulation of heat shock proteins (Hsp): Hsp70 (158) and Hsp 90 (159) and can also induce activation of UPR genes such as *XBP1* (149), or CHOP (149, 157). The importance of UPR in PRIMA-1MET cytotoxicity is further validated when knock-down of CHOP led to increased resistance to PRIMA-1MET in p53-null cells (157).

Moreover, PRIMA-1 and PRIMA-1MET were found to have antitumor effects in many cancers: lung, myeloma, breast, colorectal, cervical, ovarian, pancreatic, head and neck, thyroid, leukemia, sarcoma, prostate, liver, bladder, glioblastoma, oesophageal, melanoma, waldenstrom macroglobulinemia and oesophageal cancer (144). Their cytotoxic effects are dependent on several factors such as its dose, time since exposure, and the cellular context (i.e. confluency and hypoxia) (144). A large array of antitumor effects has been reported to a panel of the human malignancies mentioned above, including: inhibition of cell proliferation and viability, accumulation of cells in the G0/G1 and G2 phase, inhibition of colony formation and cancer cell migration (144). However, the main mechanism is to induce apoptosis of both wtp53 and mutp53 cell lines (144). Possible mechanisms that were reported in previous studies to induce apoptosis include activation of caspases (160-162), cleavage of caspase 3 (158, 163, 164) or of the poly (ADR-ribose) polymerase (PARP) (71, 154). Furthermore, PRIMA-1MET has also been shown to induce cell death through intrinsic mitochondrial apoptosis via mitochondrial release of cytochrome C (165, 166) or activation of caspase 9 (165). It is equally important to note that not all cancers show induction of apoptosis following PRIMA-1MET exposure such as in prostate cancer (167), or soft tissue sarcoma (168) suggesting that there are alternative mechanisms of cell death. For example, in mutp53 expressing soft-tissue sarcoma cell lines, autophagy was observed following PRIMA-1MET treatment (168).

Both PRIMA-1 and PRIMA-1MET were found to inhibit growth of tumor xenografts with mutp53 background and to have no cytotoxic impact in normal human cells (144). This could be explained by the fact that PRIMA-1MET was found to be cytotoxic in lung adenocarcinoma cells with high levels of mutp53 such that only cells with low mutp53 levels survived whereas, cells with high mutp53 levels were more sensitive to PRIMA-1MET (160).

1.4.3. PRIMA-1/PRIMA-1MET and its combination with other therapies

The major roles of p53 in tumor suppression and the potential of PRIMA-1MET to restore wildtype function to mutant p53, prompted several investigations to study the effects of combining PRIMA-1MET with other established therapies i.e. chemotherapies, ionizing radiation or targeted therapies to be able to treat tumors that are more aggressive due to their genetic profile or acquired resistance to treatment. Keeping in mind that ionizing radiation (IR) leads to DNA damage and activation of p53, the combination of IR and PRIMA-1MET was investigated in prostate cancer (167). Cells treated with PRIMA-1MET for 24hr with irradiation 5hrs post PRIMA-1MET treatment showed a decrease in the surviving fraction in clonogenic survival (167).

Furthermore, chemotherapeutic agents that can lead to DNA damage are believed to synergize with PRIMA-1MET in mutp53 cell lines because they trigger p53 activation. Platinum drugs such as, cisplatin was found to synergize with PRIMA-1MET in many cancers. For example, PRIMA-1MET was reported to synergize with cisplatin in colon (160), lung (160), head and neck (169), oesophageal (170), pancreatic (161) and ovarian cancers (151). This could be justified by greater availability of PRIMA-1MET to bind DNA rather than binding to glutathione and through induction of apoptosis. Another type of chemotherapeutic agent are antimetabolites (purine or pyrimidine analogs) which have been shown to synergize with PRIMA-1MET. An example of a pyrimidine antimetabolite that synergizes with PRIMA-1MET is 5-FU in head and neck (169), and oesophageal cancer (163). Fludarabin, a purine analog, showed both additive and synergistic effects when tested in chronic lymphocytic leukemia (171) and acute myeloid leukemia (152) cell lines, respectively. PRIMA-1MET was also found to increase cell toxicity with intercalating agents such as doxorubicin in breast cancer (159), and thyroid cancer (172). In addition, it synergized with other agents involved in various p53-independent mechanisms such as an ER stress inducer, tunicamycin in mutp53 myeloma cells (157) or an inhibitor of the antiporter of glutathione, sulfasalazine in mutp53 oesophageal adenocarcinoma cells (170).

Thus, PRIMA-1MET was shown to be a very promising agent that can synergize with a wide-range of therapies. Therefore, it would be very interesting to study its effects in GBM with

the goal to develop effective combination therapies to overcome major clinical challenges in GBM resistance.

1.4.4. PRIMA-1MET and GBM

As mentioned above, alterations in the *TP53* gene have been reported in 25-30% of primary GBM with an increased onset of *TP53* mutations in the proneural GBM subtype (71). These mutations may confer mutp53 GOF properties therefore, small molecules designed to restore wtp53 function have emerged as a potentially promising method to circumvent these properties in GBM. To investigate response to PRIMA-1MET for the very first time in GBM cell lines, we used isogenic cell lines for MGMT and other established cell lines with different MGMT and p53 status. In addition, given their role as drivers of tumor initiation and in resistance to chemoradiation and GBM recurrence, we also investigate response to PRIMA-1MET in brain tumor initiating (stem) cells (BTICs) with different p53 and MGMT status. Our lab showed that silencing MGMT in T98G cell lines was associated with decreased mutp53 and that PRIMA-1MET decreased cell viability in a time and dose-dependent manner and clonogenic potential in GBM cell lines irrespective of p53 (71). By testing PRIMA-1MET in cell lines isogenic for MGMT, and additional GBM cell lines with different MGMT and p53 status, we identified a positive correlation between increased MGMT expression and increased resistance to PRIMA-1MET (71). Strikingly, PRIMA-1MET disrupted the structure of neurospheres derived from BTICs and showed a dose-dependent cytotoxic effect in BTICs with a lower dose of PRIMA-1MET required to induce cell death in these cells compared to GBM established cell lines. Cytotoxicity of PRIMA-1MET in BTICs was associated with either (i) decreased expression of MGMT and activation of wtp53 in MGMT-positive BTICs or (ii) decreased expression of mutp53 in mutp53 MGMT-negative BTICs (71). PRIMA-1MET p53-independent mechanism has not yet been investigated in GBM. The role of PRIMA-1MET in alteration of redox balance has been proposed as a potential mechanism in several cancer types.

1.5. Redox Balance and Cancer

Cancer cell function is dependent on redox balance maintained by two key agents: reactive oxygen species (ROS) and antioxidants (Figure 1.12) (173). ROS are highly reactive radicals, ions or molecules that arise from partially reduced oxygen with at least one unpaired

electron in their outermost electron shell (173-175). They can be divided into two groups: free oxygen radicals (i.e. superoxide (O2•−), hydroxyl radicals (•OH), thiyl radicals(RS•), and disulfides(RSSR)) or non-radicals (i.e. hydrogen peroxide(H202), hypochloride (HOCl), singlet oxygen (1O2) and nitronium(NO2+)) (173). Among these compounds, the most studied ROS in cancer are superoxide, hydrogen peroxide, singlet oxygen, and hydroxyl radicals (175).

On the other hand, antioxidants are negative regulators of oxidative stress that protect the cell from ROS-induced damage by depleting intracellular levels of oxygen radicals (173). Examples of antioxidants include: thioredoxin, and glutathione (173). Both ROS and antioxidants are expressed in normal cells however, tumor cells possess genetic alterations that promote continuous and elevated production of ROS (176). Elevated levels of ROS in cancer cells are associated with various tumorigenesis mechanisms (177). If ROS levels increase beyond a certain threshold it can revert from tumor-promoting to inducing apoptotic signaling (Figure 1.12) (177). Therefore, to maintain and to detoxify levels of ROS in cells, cancer cells also express increased levels of antioxidants (177).

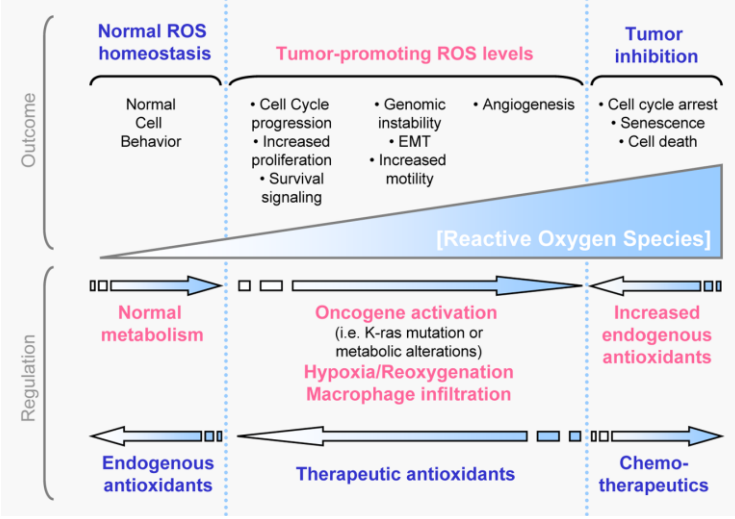


Figure 1.12. Redox balance in cancer is maintained by antioxidants and ROS. ROS has both tumor-promoting and tumor-suppressing functions. Figure adapted from (173).

ROS can increase due to many factors whether they are endogenous or exogenous. Exogenous sources of ROS include ionizing radiation, UV light, pathogens, inflammatory cytokines, and behavioral habits such as smoking (178). Major sources of endogenous ROS are hydrogen peroxide and superoxide anion generated as byproducts of cellular metabolism i.e. mitochondrial respiration (179). Moreover, ROS generation arises in the mitochondria from

incomplete coupling of electrons and H+ with oxygen in the electron transport chain (180). The endoplasmic reticulum (ER) is also a major site for cellular redox metabolism where the formation of disulfide bonds occurs (181). More specifically, the lumen of the ER contributes to 25% of ROS generation and contains a high ratio of GSSG/GSH (181). Other endogenous sources include peroxisomes and cytochrome P450 (179).

1.5.1. Functions of ROS

ROS act as a "double-edged sword", that possesses both tumor promoting functions (ex. cell cycle progression, adapting to hypoxia, increased survival and proliferation, increased motility and angiogenesis) and tumor-suppressive functions such as activation of various cell death pathways (182). The role of ROS is dependent on its source, oxidants formed and its basal concentration (183).

ROS is involved in the initiation or progression of cancer by inducing genetic instability, increasing proliferation, angiogenesis, etc. (173). Damage to DNA (both chromosomal and mitochondrial) by ROS is reported to be an important mutagenic and carcinogenic factor (184). The most common mutations of ROS are modification of guanine from G to T in both oncogenes and tumor suppressors such as p53 (184). Other types of mutations include oxidized purines and pyrimidines, formation of alkali labile sites, DNA-protein cross linkages, miscoding of the DNA lesion, gene duplication or single strand breaks/double strand (184-187). Each type of ROS induces different types of DNA damage (173). Cells that escape DNA repair are involved in cancerous growth (184).

Furthermore, ROS has direct effects on cell proliferation and apoptosis by activating various transcription factors (184, 188). For example, ROS activates the c-Jun and c-Fos subunits of AP-1 which promotes mitosis and cell division by enhancing the expression of cyclin D1 and CDKs (184, 188). It also plays a role in regulating cell motility and governing crucial steps in metastasis such as decreasing cell adhesion to the extracellular matrix (184, 188). For example, MCF-7 breast cancer cells displaying low- or non-motility showed increased motility due to increased levels of ROS and orthotopic tumors displayed metastasis to various organs i.e. lung, liver, spleen (173). Tumor cells constitutively generate high levels of H202 that act as "second messengers" increasing expression of oxidant stress factors and expression of antioxidant enzymes protecting cells from induction of apoptosis (189). Cancer cells face both hypoxia and

re-oxygenation in tumors, if cells are exposed to hypoxia for a prolonged period it can lead to cell death. To prevent this, tumor cells use the "Warburg effect", a switch to glycolysis to be able to adapt to hypoxic environment (173). Previous studies showed that oxidative stress leads to an increase in angiogenesis (173). Vascular epithelial growth factor (VEGF) is a growth factor involved in angiogenesis and its expression results in an increase in ROS through nutrient deprivation and hypoxia (173, 190). It is also reported that suppressing endogenous ROS by mitochondrial inhibitors results in a decrease in VEGF expression in cancer cells (173, 190). ROS can also promote vessel growth in the tumor microenvironment and trigger vasodilation (173).

Increase in ROS levels beyond the toxic threshold may lead to induction of cell death via apoptosis, autophagy, or necrosis (173, 190). Both the extrinsic and intrinsic apoptotic pathways are activated by ROS (191). With regards to the extrinsic pathway, ROS can activate apoptosis through the activation of transmembrane death receptors i.e. Fas, TRAIL-R1/R2 and TNF-R1 (191). For example, in the Fas pathway, once the receptor is activated, both the adaptor protein, Fas-associated death domain (FADD) and initiator procaspases-8/ -10 are recruited to the cytoplasmic surface of the receptor to form the death-inducing signaling complex (DISC) (191). This leads to the activation of caspases-8/-10 and then the activation of downstream effectors that trigger apoptosis (191). These downstream effectors can also lead to activation of the intrinsic apoptotic pathway (191). For the intrinsic pathway, ROS can activate pro-apoptotic proteins by activating the tumor suppressor, p53 and/or c-Jun N-terminal kinase (JNK) (191). It can also facilitate cytochrome c release, by opening Bax/Bak channels on the outer mitochondrial membrane, that bind to the apoptosome leading to apoptosis (191). Extremely high levels of ROS might induce necrosis, an uncontrolled cell death process and autophagic cell death through mitochondrial damage and depolarization (192, 193).

Activation of ROS signaling pathways are regulated by: Mitogen Activated Protein Kinase (MAPK) and redox-sensitive kinases (184). MAPKs are serine/threonine kinases that upon stimulation phosphorylate serine and/or threonine residues of various substrates involved in gene expression, mitosis, proliferation, motility and programmed cell death (184). Another class of ROS regulators are those that modulate downstream signal pathways using cysteine motifs as redox-sensitive sulfhydryl switches (184). This cascade involves factors such as cytoplasmic factors (i.e.thioredoxins), nuclear signaling factors (i.e. Ref-1, Redox factor-1) and transcription

factors (i.e. AP-1, NF-kB, and Egr-1) (184).

1.5.2. ROS and GBM

Although the etiology of GBM remains largely unknown, several studies have proposed alterations of redox balance to play an important role in its development(189). GBM oncogenic drivers such as, p53 and EGFR are believed to also affect redox balance (189). For example, EGFR, frequently amplified in primary GBMs, can induce production of intracellular ROS and hydrogen peroxide once EGF binds to its receptor (189). Consequently, high levels of H2O2 can increase Tyr autophosphorylation by EGFR and further augment ROS generation. As mentioned earlier, mutations of the *TP53* gene is common in secondary GBMs. p53, a protein it encodes, regulates genes involved in redox regulation i.e. mitochondrial superoxide dismutase 2 and glutathione peroxidase 1 (GPx1) (189). Furthermore, it is believed that redox imbalance can promote gliomagenesis by inducing DNA damage, cell proliferation, and resistance to treatment. (189)

The most common ROS-induced DNA modifications in the brain are –oxo-7,8-dihydroguanine (8-oxoGua) and 2,6-diamo-4-hydroxy-5-formamdopyrimidine which lead to various types of DNA damage i.e. production of apurinic/apyrimidinic DNA sites, oxidized purines and pyrimidines, single-strand DNA breaks and double-strand breaks (189). Moreover, the brain is very susceptible to the damaging effects of ROS because of its high basal metabolic activity (189). To counteract ROS stimuli, one key regulator in gliomas, is the nuclear factor erythroid 2-related factor (Nrf2), a transcription gene regulator of cellular redox homeostatsis (194, 195). In previous studies, Nrf2 was knocked down inhibited cellular proliferation (194, 195). In addition, Neurath et al. reported that glioma cells which were exposed to prolonged hypoxia resulted in increased expression of adenosine monophosphate-activated protein kinase (AMPK) and their VEGF signaling (194, 195). Furthermore, it was found that GBM cells with low levels of ROS and higher levels of total antioxidant capacity are more resistant to the chemotherapeutic agent, TMZ than those with high levels of ROS (196).

As with many other cancers, it is important to note that if levels of ROS increase to highly toxic levels this can activate various ROS-induced cell death pathways suggesting that targeting redox balance might represent a potential mechanism to eradicate GBM.

1.5.3. Thioredoxin and Thioredoxin Reductase 1, Antioxidant System in GBM

Regulation of ROS production involved an array of defensive systems including thioredoxin (Trx), a major disulfide reductase system that maintains redox balance in the brain (197).

Thioredoxin (Trx) is a small (12kDa) ubiquitous enzyme with a -CGPC- active site. Structurally, Trx is folded into four β-strands in the core and α-helices around the core (197). There exist two isoforms of Trx: one is cytosolic Trx1, which can be translocated into the nucleus and secreted out of the cell under certain circumstances whereas the other is trx2, a mitochondrial isoform (197). The thioredoxin system comprises thioredoxin-1 (Trx1), thioredoxin reductase 1(TrxR1), and NADPH (197). The NADPH-dependent TrxR1 is a pivotal enzyme in the thioredoxin system, reducing dithiol molecules of Trx1 with NADPH as an electron-donor (197, 198). Reduced Trx1 then reduces downstream proteins involved in regulating cellular redox state, inhibiting apoptosis and increasing resistance to treatment (197, 198).

TrxR1 is a redox protein in thioredoxin system associated with many different functions i.e. proliferation, redox balance, angiogenesis, changes in cell morphology and DNA repair and has been extensively studied as a potential cancer target in gliomas (199). TrxR1 is a homodimeric flavoenzyme that contains a penultimate C-terminal selenocystein in its Gly-Cys-SeCys-Gly active site (200). For both expression and activity of TrxR1, selenium is required therefore, increasing selenium increases levels its activity, and levels (200). TrxR1 is believed to be regulated by various factors. For instance, in bovine arterial endothelial cells, it is reported to be induced by cadmium which in turn stimulates the antioxidant responsive element (ARE) in the promoter of human *TXNRD1* gene encoding for TrxR1 (201). Moreover, in this study, it was also found that overexpression of the NF-E2-related factor (Nrf2), a transcription factor encoded by the *NFE2L2* gene and a master regulator of expression of antioxidant proteins, enhanced *TXNRD1* promoter activity, whereas overexpression of its dominant-negative mutant isoform suppressed cadmium-induced activation of *TXNRD1* (201). Additionally, diethyl maleate and arsenite which are activators of Nrf2, enhanced TrxR1 expression (201). In sum, these studies propose Nrf2 to be a crucial regulator of TrxR1. Moreover, it is proposed that a hypoxic environment downregulates TrxR1 mRNA and protein levels independently of hypoxia inducible factors (202). In addition, its mRNA levels are regulated post-transcriptionally through AUUUA

motifs (AU-rich elements) and its proximal promoter consists of increased GC content, and binding sites for the following transcription factors: Oct-1, Sp1 and Sp3 (202). It is reported that the *TXNRD1* fulfills the criteria of a house-keeping gene and is the first of this class to be regulated by AU-rich element regions (202).

Reduced TrxR1 targets and reduced Trx led to the activation of different proteins such as reductive enzymes (peroxiredoxin, ribonucleotide reductase and methionine sulfoxide reductase) and redox-sensitive molecules (i.e. apoptosis signal-regulated kinase 1 (ASK1), thioredoxin interacting protein and phosphatase and tensin homolog) (198). These different proteins are involved in cellular processes such as development, migration, metabolism and inflammation (198). Moreover, the thioredoxin system has been suggested to modulate sulfhydryl-disulfide isomerization reactions that regulate the conformation and functions of p53 and other redox-sensitive transcription factors i.e. NFkB and HIF, which maintain p53 in an active state (203). In Yoo et al.'s study, it was found that TrxR1-deficient cancer cells lost self-sufficiency of growth and expressed low levels of the DNA polymerase α which is important for DNA replication (199). Among these functions, the main role of TrxR1 is to maintain levels of Trx1 in a reduced state (197, 198).

Through analysis of 433 astrocytomas, TrxR1 was found to be upregulated in more than 66% of the cases and to be associated with increased malignancy and recurrence rate (204). Levels of TrxR1 was significantly higher in GBM tissue than normal brain tissue and TrxR1 levels in the blood is also higher in GBM patients relative to controls (205). This suggests that TrxR1 may be involved in GBM progression. Overexpression of TrxR1 in GBM cell lines, U87MG and T98G significantly enhanced radioresistance (206). As well, knockdown of *TP53* induced glycolysis and apoptosis regulator knockdown is associated with radiosensitization of cell lines by inhibiting IR-induced TrxR1 nuclear transport (206). Given the role of TrxR1 in GBM and its increased levels in GBM tumor tissues of patients newly diagnosed with GBM, targeting TrxR1 represents a potential strategy to treat these patients.

1.5.3.1 Inhibitors of Thioredoxin Reductase 1 (TrxR1)

Current pharmacological strategies to treat cancer include taking advantage of the dependency of tumor cells on antioxidants by using agents that can dampen antioxidant systems (173). Moreover, inhibition of antioxidant systems is reported to be an effective therapy to

sensitize cells to apoptotic cell death (173). The efficacy of many anti-cancer therapies is dependent on its effect in inducing ROS production (173). As TrxR1 has been implicated in many functions associated with cancer promotion, inhibition of TrxR1 is being extensively studied. There are various types of inhibitors of TrxR1 including those that bind to the NADPH binding sites or to the Cys or Sec residues in the redox site of the enzyme, which covalently modifies the reactive sites of the enzyme (207). Various anticancer agents currently used in the clinic have been implicated in the inhibition of TrxR1 by potentially reacting with the Sec residue in the active site of TrxR1, such as platinum chemotherapy derivatives (i.e. cisplatin), nitrogen mustards (i.e. chlorambucil), arsenic trioxide and gold compounds (i.e. auranofin) (208). Auranofin is a promising irreversible inhibitor of TrxR1 for cancer treatment as it has been already approved by the FDA for the treatment of rheumatoid arthritis (209).

As mentioned earlier, it has been recently proposed that the active metabolite of PRIMA-1MET, methylene quinuclidinone can also bind to TrxR1 and therefore, inhibits its expression in lung adenocarcinoma and osteosarcoma cells (210). Thus, the potential of PRIMA-1MET to have alternative mechanisms to mediate cytotoxic effects in GBM makes it a promising agent to test in GBM and to further understand its mechanism.

1.6. Connecting Text: Rationale, Hypothesis and Objectives

Despite multimodal treatment, patients diagnosed with GBM have a low survival rate of 14.6 months (20). This has been primarily related to chemoradiation resistant mechanisms such as, MGMT expression, which is of clinical relevance as an important predictor of TMZ cytotoxicity (34). Interestingly, previous studies showed that wtp53 negatively regulates MGMT expression (65) and several studies showed that missense mutations of the *TP53* gene confer novel oncogenic properties (106). Therefore, small molecules that can circumvent oncogenic properties of mutp53 and revert mutp53 to its wildtype form represent a promising strategy to treat patients diagnosed with GBM. Among the small molecules discovered to reactivate mutp53 to its wild-type form and that is being extensively studied in various cancers alone or in combination with other therapies is PRIMA-1MET (144).

Recently, our lab investigated the effects of PRIMA-1MET for the very first time in GBM (71) and reported a novel mechanism to circumvent mutp53/GOF activities through decreased expression of MGMT in GBM established cell lines mutp53 and silenced for MGMT (71). Furthermore, testing PRIMA-1MET in T98G MGMT isogenic cell lines, other human established cell lines (U87MG, LN-18, U138, and A172) and brain tumor initiating (stem) cells (BTICs) with different MGMT and p53 status showed PRIMA-1MET to be preferentially cytotoxic in a dose and time-dependent manner in MGMT-negative cells irrespective of p53 status (71). While these findings suggested for the first time the role of MGMT in resistance to PRIMA-1MET (71), they raise questions with regards to the mechanisms underlying p53-independent effects of PRIMA-1MET and MGMT-mediated resistance to PRIMA-1MET in GBM. This study therefore aims to investigate the above-mentioned questions.

In GBM, PRIMA-1MET showed differential effects on GBM established cell lines irrespective of p53 status and based on expression of MGMT (71). Furthermore, in many other studies, PRIMA-1MET was shown to increase levels of reactive oxygens species (ROS) and decrease levels of antioxidants apart from its effects on mutp53 (144). For instance, in adenocarcinoma and osteosarcoma cells, PRIMA-1MET decreased levels of TrxR1 irrespective of p53 status (210). As both MGMT, a DNA repair protein and TrxR1, an antioxidant enzyme, have been associated with p53-independent mechanisms of PRIMA-1MET, we sought to investigate the relationship between MGMT and TrxR1 and whether MGMT confers resistance to PRIMA-1MET by modulating redox balance. Therefore, we hypothesized that MGMT-

mediated modulation of redox balance and the relationship between MGMT and TrxR1 might account for p53-independent cytotoxic effects of PRIMA-1MET in GBM.

Therefore, as outlined below, the main aims of this thesis were:

1) To investigate PRIMA-1MET effects on redox balance as a potential p53-independent mechanism in GBM.
2) To elucidate the mechanisms underlying the differential effects of MGMT with respect to cytotoxicity of PRIMA-1MET in GBM.

Chapter 2.

Manuscript in preparation for submission to Scientific Reports (Nature Publishing Group)

Interplay between MGMT and Thioredoxin Reductase 1 modulates PRIMA-1MET (APR-246)-induced cytotoxicity in Glioblastoma

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2.1 Abstract

Glioblastoma multiforme (GBM), the most common and advanced primary brain malignancy in adults remains an incurable disease, despite aggressive treatment with surgery, radiation therapy and chemoradiation using the alkylating agent, Temozolomide (TMZ). PRIMA-1MET (APR-246), a small molecule designed to restore mutant (mut)p53 function has been shown to affect cellular redox status through targeting thioredoxin reductase 1 (TrxR1) in wild-type (wt)p53 cancer cells. We have recently shown that PRIMA-1MET exerts cytotoxic effects status preferentially in GBM cell lines expressing low levels of the DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT), known for its role in resistance to TMZ. We hypothesized that PRIMA-1MET mediates its growth inhibitory effects by modulating the redox balance and investigated the potential relationship between MGMT, redox balance and TrxR1. We show that PRIMA-1MET decreased TrxR1 expression levels preferentially in MGMT-low expressing isogenic GBM cell lines. Using pharmacological agents that modulate reactive oxygen species (ROS) levels i.e., ROS scavenger, N-acetylcysteine and ROS inducer, L-Buthionine-Sulfoximine, we show that PRIMA-1MET exerts its growth-inhibitory effects through increased ROS. Strikingly, we identified a novel positive relationship between MGMT and TrxR1, wherein high MGMT expression is associated with high expression of TrxR1 and low levels of ROS. Treatment with the MGMT inhibitor, O6-Benzylguanine, or the TrxR1-targeting FDA-approved drug Auranofin validated our findings. Interestingly, the latter exerted significantly more pronounced cytotoxic effects compared to PRIMA-1MET in GBM cell lines. Additional studies are warranted to assess PRIMA-1MET in combination with TrxR1-targeting therapies and propose repurposing of Auranofin as a novel strategy to improve the dismal outcome of patients MGMT-positive GBM.

2.2 Introduction

Glioblastoma Multiforme (GBM), a common invasive astrocytic glioma accounts for approximately 54% of all brain tumors in adults (1). Despite the stratification of GBM into four distinct molecular signatures (2), all patients are currently treated with surgery, concomitant radiotherapy (RT) and chemotherapy using the alkylating agent, Temozolomide (TMZ) followed by adjuvant TMZ (3). However, the prognosis of patients diagnosed with GBM remains extremely poor with an average survival of 14.6 months(3).

The DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT) repairs the mutagenic DNA adduct, O6-methylguanine conferring resistance to TMZ (4). Intriguingly, MGMT was found to interact with over 60 proteins associated with important biological functions, including cell cycle progression, DNA replication and translation and might be involved in other functions beyond its role in DNA repair (5).

Alterations in the *TP53* gene a potent tumor suppressor and transcription factor have been reported in 25-30% of primary GBM (1). Activation of p53 in response to genotoxic stress (i.e. DNA damage induced by irradiation, hypoxia, chemotherapeutic drugs) (6, 7) leads to the induction of various tumor-suppressive pathways (6, 7). Mutations of the *TP53* gene are often missense mutations that confer mutant (mut)p53 oncogenic "gain-of-function" GOF properties (8). Small agents that either reactivate mutp53 to its wildtype (wt) conformation or destabilize mutp53 have been proposed as an effective way to circumvent mutp53-mediated oncogenic functions. PRIMA-1 (p53 reactivation and induction of massive apoptosis) and its more active and methylated derivative, PRIMA-1MET reactivate mutp53 by physically interacting with p53 and restoring its wtp53 conformation and transcriptional activity (9). Recent studies have also shown that PRIMA-1MET is also effective in wtp53 cancers (10) through the induction of reactive oxygen species (ROS) and inhibition of antioxidants by its active metabolite, methylene quinuclidinone (10).

Antioxidants, i.e. thioredoxins are important regulators of ROS (11, 12). Thioredoxin reductase 1 (TrxR1), a key redox protein in the thioredoxin system is elevated in tissue and blood of GBM patients (12). The potential of PRIMA-1MET to exert multiple mechanisms (10) prompted our previous investigation showing cytotoxicity of PRIMA-1MET for the first time in GBM cell lines and patient-derived GBM stem cells (GSCs) preferentially in GBM-expressing low levels of MGMT irrespective of p53 status (13).

In the present work, we investigated whether PRIMA-1MET affects proliferation in GBM cell lines isogenic for MGMT through modulation of ROS levels and inhibition of TrxR1 irrespective of p53 status. We also investigated the role of MGMT in resistance to PRIMA-1MET with respect to redox balance and TrxR1 levels in GBM. Using wtp53 and mutp53 isogenic cells for MGMT, we demonstrate that PRIMA-1MET increased ROS while decreasing TrxR1 expression levels in GBM based on MGMT status and identified a novel relationship between MGMT and TrxR1. We propose an alternative method to circumvent MGMT resistance by combining PRIMA-1MET with TrxR1-targeting agents such as, Auranofin, an irreversible inhibitor of TrxR1.

2.3 Results

2.3.1 PRIMA-1MET increases intracellular reactive oxygen species in GBM cell lines irrespective of p53 status

We have previously shown that PRIMA-1MET exerts cytotoxic effects on human GBM cell lines and GSCs irrespective of p53 status (13). Cytotoxic effects of PRIMA-1MET are mediated through induction of reactive oxygen species (ROS) in different wtp53 cancer cell lines including acute myeloid leukemia (14), epithelial ovarian cancer (15), and myeloma (16). Significant increase in ROS has been associated with tumor-suppressive activities through the induction of various cell death pathways (17). To investigate the hypothesis that PRIMA-1MET induces ROS in GBM, we assessed induction of intracellular ROS by PRIMA-1MET (24-hour treatment) using 5-6-chloromethyl-2'7'-dichlorodihydroflorescein diacetate, acetyl ester (CM-H2DCFDA) in GBM cells with known p53 status and MGMT expression levels. We used GBM established cell lines isogenic for MGMT i.e. knockdown (mutp53 T98/EV and its counterpart T98/shRNA) and overexpression models (wtp53 U87/EV and its counterpart U87/MGMT) in addition to LN-18, mutp53 (C23S substitution) constitutively expressing high endogenous levels of MGMT protein (Figure 2.1A). Cells were treated with PRIMA-1MET for 24hrs at 25μM, and 50μM and levels of ROS were quantified using a fluorescent microplate reader. PRIMA-1MET (25μM) induced significant ROS in MGMT-negative cell lines U87/EV and T98/shRNA cell lines (1.9±0.3 and 1.5±0.1fold change) relative to their respective DMSO controls, irrespective of p53 status (Figure 2.1B). Treatment with PRIMA-1MET (25μM) did not significantly increase ROS levels in MGMT-positive cell lines U87/MGMT, T98/EV, and LN-18 (1.1±0.1, 1.0±0.04 and 1.2±0.1fold change) relative to their respective DMSO controls. Treatment with PRIMA-1MET showed a similar pattern of increased ROS levels in MGMT-negative cell lines. Taken together, these findings provide evidence for the induction of ROS by PRIMA-1MET preferentially in GBM MGMT-negative cell lines irrespective of p53 status.

2.3.2 Modulation of ROS via pharmacological agents affects PRIMA-1MET-mediated cytotoxicity in GBM cell lines irrespective of p53 status

Having demonstrated that PRIMA-1MET induces ROS in GBM cell lines, we next sought to investigate whether induction of intracellular ROS is essential for PRIMA-1MET-mediated

growth-inhibitory effects. To do so, we used ROS scavenger, N-acetylcysteine (NAc), and a ROS inducer that inhibits glutathione system, L-Buthionine-Sulfoximine (L-BSO). Cells were treated with PRIMA-1MET at doses ranging between 0-300 μM alone, with 1mM of NAc or 10μM of L-BSO for 72hrs and then we assessed the effect of PRIMA-1MET and its combination with redox modulators using MTT proliferation assay. Treatment with redox modulators at the concentrations used did not affect cellular proliferation in isogenic cell lines (data not shown).

In agreement with previous studies, we found that co-treatment of PRIMA-1MET with NAc significantly increased IC50, whereas co-treatment with L-BSO significantly decreased IC50 values in all cell lines (Figure 2.2, Table 2.1). The IC50s after treatment with PRIMA-1MET are as follows: U87/EV- 43±2μM, U87/MGMT - 84±7μM, T98/EV - 121±12μM, and T98/shRNA - 32±1μM. Co-treatment with NAc significantly increased the IC50s for each cell line compared to PRIMA-1MET alone (U87/EV: 166±7μM, U87/MGMT: 164±11μM, T98/EV: 205±14μM, and T98/shRNA: 152±10μM). The IC50s for each cell line after co-treatment with L-BSO are: U87/EV: 10±7μM, U87/MGMT: 34±3μM, T98/EV: 65μM, and T98/shRNA: 20±1μM. The fold-change relative to control for each cell line following their respective co-treatment (NAc or L-BSO) are as follows: U87/EV (NAc: 3.8±0.2, L-BSO: 0.3±0.2), U87/MGMT (NAc: 2.0±0.3, L-BSO: 0.4±0.03), T98/EV (NAc: 1.7±0.2, L-BSO: 0.6±0.1) and T98/shRNA (NAc: 4.7±0.4, L-BSO: 0.6±0.05) (Figure 2.2, Table 2.1). Hence, NAc co-treatment showed a greater effect in MGMT-negative cell lines (U87/EV: 3.8 and T98/shRNA: 4.7) compared to MGMT-positive cell lines (U87/MGMT: 2.0 and T98/EV: 1.7), whereas the effect of L-BSO was relatively the same between isogenic cell lines (U87/EV vs. U87/MGMT: 0.3 vs. 0.4; T98/EV vs. T98/shRNA: 0.6 vs. 0.6) (Figure. 2.2, Table 2.1).

Taken together, these results show that induction of ROS plays an important role in the cytotoxic effects of PRIMA-1MET irrespective of p53 status, and suggest a potential link between MGMT and ROS in GBM.

2.3.3 PRIMA-1MET decreased TrxR1 expression levels in GBM cell lines irrespective of p53

Next, we sought to dissect the potential mechanism by which PRIMA-1MET induces ROS in GBM cell lines. Recently, Peng et al. (2013) found that the active metabolite of PRIMA-1MET,

methylene quinuclidinone (MQ) inhibits TrxR1 in lung adenocarcinoma and osteosarcoma cells (18). TrxR1, a key redox protein involved in the thioredoxin system (19) is one of two major antioxidant systems in the brain that regulate levels of ROS (20). To investigate whether PRIMA-1MET inhibits TrxR1 in GBM cell lines, we used Western blotting to assess expression levels of TrxR1 after treatment with PRIMA-1MET (25μM, and 50μM for 24h). Densitometric analysis showed that all cell lines had a dose-dependent decrease in TrxR1, except for U87/MGMT which required a higher concentration of PRIMA-1MET to decrease TrxR1 levels (Figure 2.3). Additionally, PRIMA-1MET-induced decrease in TrxR1 expression was more pronounced in MGMT-negative cell lines compared to their MGMT-positive counterparts irrespective of p53 status. As shown in Figure 2.3, MGMT-positive cell lines (U87/MGMT, T98/EV and LN-18) had a higher expression of TrxR1 compared to MGMT-negative cell lines (U87/EV and T98/shRNA). Hence, PRIMA-1MET decreased expression levels of TrxR1 in both mutp53 and wtp53 cell lines with differential effects based on expression of MGMT in GBM.

2.3.4 Differential Expression of TrxR1 and ROS in GBM cell lines isogenic for MGMT

We showed that both MGMT and TrxR1 are involved in PRIMA-1MET cytotoxicity irrespective of p53 suggesting that MGMT-positive cell lines display higher expression of TrxR1 compared to their MGMT-negative counterpart. We subsequently investigated whether there is a causal link between MGMT and TrxR1 by Western blotting to assess TrxR1, MGMT and p53 protein levels in MGMT-knockdown and overexpression isogenic GBM cell lines and in LN-18 cell line (mutp53 and high levels of MGMT).

In T98 mutp53 MGMT isogenic cell lines, a significant decrease in TrxR1 levels was found when MGMT was knockdown by a short-hairpin (sh)RNA in comparison to its empty vector counterpart. (Figure 2.4A) Furthermore, when MGMT was overexpressed in U87MG wtp53 cell line, we found a slight increase in TrxR1. (Figure 2.4A) This confirms that increased MGMT expression is associated with increased TrxR1 levels. In addition, mutp53 cell lines have higher levels of TrxR1 relative to wtp53.

Having demonstrated a positive correlation between MGMT expression and levels of TrxR1 and given the role of TrxR1 as an important regulator of ROS, we next investigated whether there is a differential expression of ROS based on MGMT levels. To explore this, we used CM-H2DCFDA to quantify basal intracellular ROS levels. As shown in Figure 2.4B, MGMT-

positive cell lines had significantly lower levels of ROS compared to their MGMT-negative cell lines counterparts (U87/EV (21248±1508) vs. U87/MGMT (13148±728.2): $p<0.05$; T98/EV (11149±1424) vs. T98/shRNA (22587±1865): $p<0.01$) while LN-18 expressed levels of ROS at 9889±909.9, which was similar to T98/EV and U87/MGMT. Representative fluorescence images of ROS levels in T98G isogenic cells illustrate this association as the MGMT-negative cell line T98/shRNA had significantly higher expression of ROS compared to its MGMT-positive counterpart (Figure 2.4C).

To further validate that silencing MGMT is indeed associated with decreased TrxR1 levels, we depleted MGMT protein levels using O6-BG (20 μM, 48hrs), an MGMT inhibitor, for 48hrs. (Figure 2.4D) Compared to the DMSO control, MGMT-positive cells with depleted MGMT levels following O6-BG treatment showed a greater decrease in TrxR1 compared to cells with low basal MGMT levels (Figure 2.4D). Thus, these results indicate a possible link between MGMT and TrxR1 where high MGMT expression is associated with high TrxR1 expression levels and vice versa. As well, they demonstrate a potential relationship between levels of MGMT and ROS in GBM, such that cell lines with high MGMT have low levels of ROS whereas, cells with low MGMT levels display high levels of ROS.

2.3.5 TrxR1-targeting drug, Auranofin decreased proliferation differentially based on MGMT expression in GBM cell lines isogenic for MGMT

We showed that the differential effects of PRIMA-1MET in cell lines based on MGMT levels may be associated with differences in TrxR1, which is differentially decreased by PRIMA-1MET in MGMT-positive vs. negative cell lines. To further investigate the potential relationship between levels of MGMT and TrxR1. we assessed the effects of Auranofin, a well-known FDA-approved TrxR1 inhibitor in GBM cell lines isogenic for MGMT. We hypothesized that if MGMT isogenic cell lines have differential levels of TrxR1, Auranofin might have differential growth inhibitory effects in these cell lines and display similar effects seen with PRIMA-1MET. Cells were treated with Auranofin at doses ranging between 0-50 μM for 72hrs and its effects were investigated using MTT proliferation assay.

Our results indicate striking differential cytotoxic effects of Auranofin based on MGMT expression (Figure 2.5A). The mean IC50s for each cell line after treatment with Auranofin is as follows: U87/EV- 3.6±1.1μM (mean values ± SEM, N=3), U87/MGMT – 8.3±0.9μM (N=3),

T98/EV – 7.7±1.2μM (N=4), and T98/shRNA – 2.1±0.7μM (N=4). The difference in isogenic cell pairs were significantly different between both models (U87/EV vs. U87/MGMT: $p<0.05$, and T98/EV vs. T98/MGMT: $p<0.05$). Interestingly, as shown with PRIMA-1MET, both MGMT-negative cell lines had similar sensitivity to Auranofin irrespective of p53 status. Auranofin was found to be more potent in these cell lines compared to PRIMA-1MET with IC50s ranging between 1-10μM, whereas cells treated with PRIMA-1MET had IC50s between 30-125μM. Figure 2.5B illustrates resistance of MGMT-positive cells to Auranofin (5μM, for 48hrs) compared to MGMT-negative cells, which showed round, sparse and floating cells.

These results further validate that MGMT isogenic cell lines express differential levels of TrxR1 and that cytotoxicity of Auranofin is based on MGMT expression status in GBM cell lines.

2.4 Discussion

In the present study, we provide new evidence suggesting that PRIMA-1MET decreased expression of TrxR1 and induced ROS in GBM and that MGMT exerts functional roles beyond its previously described role as a DNA repair protein. We elucidated the mechanisms underlying p53-independent of PRIMA-1MET and resistance of MGMT-positive human established GBM cell lines recently reported by our group (13). Although PRIMA-1MET was initially described to restore wild-type function to mutp53, it was later reported to possess p53-independent mechanisms in various cancer models (10). Prevailing data suggests that this may be achieved through modulation of redox balance (10). The relationship between MGMT, TrxR1 and ROS is in line with a previous study reporting that MGMT exhibits other roles beyond its known function to remove cytotoxic O6-methylguanine adducts by interacting with over 60 MGMT-binding partners involved in cell cycle progression, DNA replication, translation etc. (5).

Our results indicate that PRIMA-1MET induces ROS irrespective of p53 (Figure 2.1B), which is in accordance with current studies showing similar effects in acute myeloid leukemia (14), epithelial ovarian cancer(15) and myeloma(16). Interestingly, our results reveal that MGMT-positive cells (U87/MGMT, T98/EV, and LN-18) did not induce significant ROS relative to DMSO control at 25μM or 50μM. It is well known that excessive levels of ROS are associated with activation of different cell death pathways (i.e. apoptosis) (21). These findings therefore, correlate well with the increased cell sensitivity to PRIMA-1MET we observed in MGMT-negative cells in our previous study (13) and further demonstrated in wtp53 U87/EV and U87/MGMT isogenic cell lines in the current study.

To ascertain the importance of ROS in p53-independent mechanism of PRIMA-1MET, we co-treated cells with either PRIMA-1MET with N-acetylcysteine, a ROS scavenger or PRIMA-1MET with L-Buthionine Sulfoximine, a ROS inducer that inhibits glutathione (an antioxidant). Our data show that co-treatment with NAc resulted in a significant increase in IC50s for each cell line (Figure 2.2, Table 2.1). Surprisingly, the IC50s following co-treatment were in a similar range regardless of both p53 and MGMT status. Furthermore, co-treatment with L-BSO at a concentration that does not affect cell growth decreased IC50s (Figure 2.2, Table 2.1). These results suggest that the inhibition of glutathione enhances PRIMA-1MET-induced cytotoxic effects. Our findings provide evidence for the importance of ROS in p53-independent

mechanism of PRIMA-1MET. These results also suggest a potential role of ROS in MGMT-based differential effects of PRIMA-1MET as treatment with ROS scavenger removed the drastic differences in IC50s between MGMT isogenic cell lines.

A study conducted by Peng et al. demonstrated that PRIMA-1MET inhibits TrxR1 via its active metabolite, methylene quinuclidinone (MQ) in lung adenocarcinoma and osteosarcoma cells irrespective of p53 status (18). In accordance with these findings, we report a dose-dependent decrease in TrxR1 expression in all GBM cell lines (U87/EV, T98/EV, T98/shRNA and LN-18) except U87/MGMT which based on our preliminary experiments required a higher dose of PRIMA-1MET to decrease levels of TrxR1 (data not shown). Our results also revealed that PRIMA-1MET had a more pronounced decrease in TrxR1 levels in cell lines with low MGMT expression compared to high MGMT expression irrespective of p53 status (Figure 2.3A). Interestingly, when comparing the control conditions of isogenic cell lines, we saw a difference in TrxR1 expression such that cells with high MGMT expression (U87/MGMT and T98/EV) have a high expression of TrxR1 and vice versa.

We showed that ROS levels correlate negatively with levels of TrxR1 and MGMT. MGMT-positive cells which did not induce significant ROS had high expression of TrxR1, whereas the MGMT-negative cell lines which had a more pronounced decrease in level of TrxR1 expression showed significant induction of ROS at 25μM (Figure 2.3B). This is in agreement with the study conducted by Peng et al., which showed that binding of MQ to TrxR1 is crucial for induction of ROS (18). However, in these same cells, at concentration of 50μM, there was no significant decrease in ROS relative to control. This effect could be explained by the fact that these cells have relatively low IC50s, therefore, cell death may be a confounding factor for assessing the levels of intracellular ROS (Figure 2.2A). Taken together, our data are consistent with findings showing decrease of TrxR1 expression levels and induction of ROS as major p53-independent mechanisms currently proposed in other cancer types (10).

Differentials levels of TrxR1 and induction of ROS observed in MGMT isogenic cells after PRIMA-1MET treatment support a relationship between MGMT and redox balance. We quantified expression of TrxR1 in MGMT isogenic cell lines, both wtp53 and mutp53 and found an association between MGMT and TrxR1 such that isogenic cell lines with high MGMT levels

had a significantly higher expression of TrxR1 compared to their negative counterparts (Figure 2.4A). In accordance with our previous results, we found MGMT silencing to be associated with decreased mutp53 expression and TrxR1 (13) (Figure 2.4A). p53 is associated with redox balance (22), therefore whether wtp53 or mutp53 is involved in the relationship between MGMT and TrxR1 needs to be further investigated. We further validated this relationship by treating isogenic cell lines and LN-18, a parental cell line that constitutively expresses MGMT with MGMT inhibitor, O6-BG (Figure 2.4D). After treatment, a decrease in TrxR1 was observed in all MGMT-positive cell lines suggesting a novel role of MGMT in regulating oxidative stress through regulation of TrxR1. It is important to note that the decrease in TrxR1 following O6-BG treatment is not as drastic as what we found when silencing MGMT in T98/shRNA using siRNA strategy. O6-BG did not completely deplete MGMT suggesting that a low threshold level for MGMT is sufficient for its interplay with TRX1 or other potential intermediate factor(s). The differential expression levels of TrxR1 support our earlier observations wherein co-treatment of PRIMA-1MET with L-BSO did not completely suppress MGMT-based differences. This could be explained by ROS being regulated by various antioxidant defense mechanisms i.e. glutathione, thioredoxin, etc. (11). Therefore, inhibition of only glutathione alone may not be sufficient to remove MGMT-based growth inhibitory effects of PRIMA-1MET.

Upregulation of TrxR1 has been associated with protecting the cells from elevated levels of ROS (23). For instance, Peng et al. showed that Sec-containing TrxR1 are required for elimination of glucose-derive hydrogen peroxide, H2O2 in mouse embryonic fibroblasts (24). In addition, pharmacological inhibition of TrxR1 via Auranofin (25-27) and RITA (23) promote elevated levels of ROS. In contrast to these studies, knockdown of TrxR1 was not sufficient for inducing ROS due to the presence of alternative antioxidant defense systems (28). This raises the question of whether other antioxidants may be differentially expressed based on MGMT. A potential candidate is glutathione, another important redox system in the brain (20). Both thioredoxin and glutathione system are part of the same family and therefore, regulated in a similar manner (20). Furthermore Du et al. demonstrated that glutathione and the thioredoxin system can reduce Trx1 if TrxR1 activity is inhibited (29). Therefore, further investigation is needed to understand the pathway by which increased MGMT decreased ROS in GBM.

To further validate our findings regarding the relationship between TrxR1 and MGMT, we

tested Auranofin, an inhibitor of TrxR1, and showed its differential effects in these MGMT isogenic cell lines with strikingly low IC50s compared to PRIMAMET (Figure 2.5). This therefore, confirms our findings that MGMT-based differences are due to the differential levels of TrxR1, its target. Thus, a potential treatment paradigm to increase PRIMA-1MET potency in MGMT-positive cell lines would be to combine PRIMA-1MET with Auranofin. Both these compounds have already been found to be synergistic in breast cancer cells (30) as many of the mechanism of action of Auranofin complements those of PRIMA-1MET such as alteration of mitochondrial function (31), generation of ROS (26, 27), inhibition of TrxR1(26, 27), and activation of caspases (27).

Our results therefore, suggest that MGMT plays a major role in maintaining a specific redox environment in the cells. Previous studies report that moderate levels of ROS contribute to tumor development however, excessive levels of ROS production beyond a toxic threshold can produce abnormal stress leading to cell death (11). Thus, the fact that MGMT-positive cells have an environment with low ROS levels and high TrxR1 expression whereas, MGMT-negative levels have high ROS and low TrxR1 suggest that these cells are constitutively at different levels of the ROS spectrum. MGMT-negative cells are closer to the toxic threshold relative to their MGMT-positive counterpart, which could ultimately explain their increased sensitivity to PRIMA-1MET. This exciting finding could be extended to other treatment modalities, especially radiation therapy known to induce ROS and DNA damage. The differential levels of ROS and antioxidants based on MGMT may possibly explain why patients with tumors harboring *MGMT* promoter methylation had better outcomes following radiation treatment (32). *MGMT* promoter methylation is correlated with low levels of antioxidants and high reactive oxygen species suggests that these patients will be more sensitive to radiation therapy and reach ROS threshold with lower doses.

Taken together, our findings reveal that PRIMA-1MET mediates its p53-independent effects through modulation of redox balance, specifically through the inhibition of TrxR1 and induction of ROS (Figure 2.6). Furthermore, we provide evidence that MGMT-based effects of PRIMA-1MET are due to the differential basal levels of TrxR1and ROS in MGMT isogenic cell lines (Figure 2.6). To our knowledge, this is the first study to propose a relationship between MGMT and TrxR1, therefore challenging the paradigm that MGMT functions only as a DNA repair

protein in GBM. However, the exact mechanism by which these two protein interact has yet to be investigated. To further investigate the relationship between MGMT and TrxR1, we need to validate our findings in a larger GBM panel, which also includes GBM stem cell cells and available public databases i.e. TCGA, NCL-60, etc. The fact that PRIMA-1MET can modulate redox balance through increased ROS in GBM provides the foundation for further development of this promising agent in GBM treatment. We showcase the potential role of MGMT in the efficacy of TrxR1-targeting drugs in the context of GBM and shed light into possible strategies that can be used to overcome MGMT resistance such as repurposing the use of Auranofin to reduce levels of TrxR1 and sensitize MGMT-positive GBM to PRIMA-1MET treatment. Thus, identification of the novel association between MGMT and TrxR1 may be of clinical relevance to understand the multifaceted role of MGMT in GBM and develop effective therapeutic strategies to improve the dismal outcome of patients diagnosed with GBM.

2.5 Materials and Methods

2.5.1 Cell Culture and Drug Treatment

Human established cell lines, U87MG, T98G, LN-18, A172, U138 GBM cell lines were obtained from the American Type Culture Collection. To assess the effect of the drugs with respect to MGMT levels, we used T98G-mutp53 and U87MG-wtp53 isogenic cell lines for MGMT. For the T98G-mutp53 model, T98G cells were transfected with either a plasmid vector encoding shRNA against MGMT (T98/shRNA) or with empty vector (T98/EV). U87MG-wtp53 isogenic model was transfected with a plasmid carrying exogenous MGMT (U87/MGMT) or the empty vector (U87/EV). All established cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine (FBS; standard medium) and 1% Penicillin/Streptomycin at 37°C and 5% CO_2. U87MG transfected cell lines were maintained in media supplemented with 1% G418 antibiotic. Cells were treated with PRIMA-1MET (Tocris Bioscience, Bristol, UK) dissolved in DMSO at varying times and doses in standard media depending on the assay. DMSO was used as a control. Cells were treated in standard medium with either N-Acetylcysteine (Sigma-Aldrich, St. Louis, MO) dissolved in PBS and prepared in standard media at a concentration of 1mM, or L-Buthionine-sulfoximine (Sigma-Aldrich, St. Louis, MO) dissolved in PBS at a concentration of 10μM, or various concentrations of Auranofin (Cayman Chemical Company, Ann Arbor, MI) dissolved in DMSO.

2.5.2 MTT Assay

Cell proliferation was assessed using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific Inc., Waltham, Massachusettes, USA). Briefly, cells were seeded in 96-well plates in standard medium and allowed to adhere overnight at 37°C in 5% CO_2. Cells were then treated with DMSO, various concentrations of Auranofin, or PRIMA-1MET alone or in combination with N-Acetylcysteine or L-Buthionine-Sulfoximine for 72hrs. Next, MTT 3-(4,5-dimenthylthiazol-2-yl)-2,5- diphenyltetrazolium bromide was added to each well followed by sodium dodecyl sulfate (SDS) 4 hours after adding MTT. The absorbance was read at 570nm after overnight incubation. Blank controls were subtracted and cell % relative to control was calculated.

2.5.3 Western Blot (WB) Analysis

Cells were first treated with either PRIMA-1MET or O6-BG for the specified concentrations and duration. After treatment, cells were washed twice with cold 1X phosphate-buffered

saline(PBS) and lysed with cold 1X RIPA buffer ((50mM Tris, 150mM NaCl, 5mM EDTA, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 50mM NaF); Boston BioProducts, Inc., Ashland, MA, USA) supplemented with 0.2mM sodium orthovanadate protease (Sigma-Aldrich, Oakville, ON, Canada) and phosphatase inhibitor cocktails (Roche Diagnostics, QC, Canada).

Proteins (30μg, based on BCA protein assay kit, Pierce) were separated by electrophoresis in 12% SDS-PAGE and transferred onto polyvnylidine difluoride (PVDF) membrane. Membranes were subsequently blocked with 5% milk in Tris-buffered saline with 0.1% tween 10 (TBS/T) for 1hr. Membranes were then probed to assess changes in expression levels of MGMT (Santa Cruz, Dallas, TX, USA), TrxR1 (Sigma-Aldrich), mutant and wtp53 (DO-1, Santa Cruz), and β-actin (Sigma- Aldrich, Oakville, ON, Canada) followed by incubation with horseradish peroxidase-conjugated goat anti-mouse antibody (Life Technologies, Carlsbad, California, USA). Amersham ECL Western Blotting Detection Reagent purchased from GE Healthcare Life Sciences (Mississauga, ON, Canada) was used to assess HRP activity by chemiluminescence. The intensities of the bands were determined by densitometry using ImageJ software analysis (National Institutes of Health, Bethesda, MD, USA). *β*-Actin was used as a loading control and all data were normalized to these loading controls.

2.5.4 Assessment of ROS Levels

At the end of the specified treatment, ROS measurement was performed using CM-H2DCFA (5-(and 6-)-chloromethyl-2',7'-dichlorodihydrofluoresceine diacetate; Invitrogen Molecular Probes, Eugene, OR). In brief, cells were seeded in 96-well plates and allowed to adhere overnight. After drug treatment for the specified time, media was removed and cells were loaded with 10μM CM-H2DCFA diluted in clear media for 30min at 37 °C. H202 was used as a positive control. Cells were then washed twice with PBS, left in clear media and fluorescence intensity was measured using a fluorescent microplate reader at an excitation of 490nm and emission of 525nm (Tecan, Infinite M2000). Relative ROS production (measured by fluorescence intensity) was calculated as follows: $(F_{drug\ treated} - F_{blank})/ (F_{control} - F_{blank})$. Basal levels of ROS in T98 isogenic cell lines were visualized with an inverted microscope (Zeiss Axio Vert.A1, Germany).

2.5.5 Statistical Analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software Inc, La Jolla, CA, USA). IC50s were determined by generating best-fit sigmoidal dose response curves

and two-tailed Student's t-test was performed to compare means as appropriate. All data are expressed as mean+/- the standard error of the mean. Probability values <0.05 were considered statistically significant.

2.6 Acknowledgements

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2.7 References

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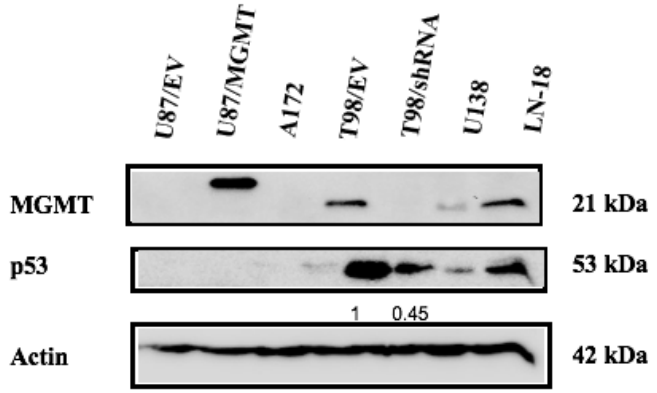
2.8 Figures

Figure 2.1

A.



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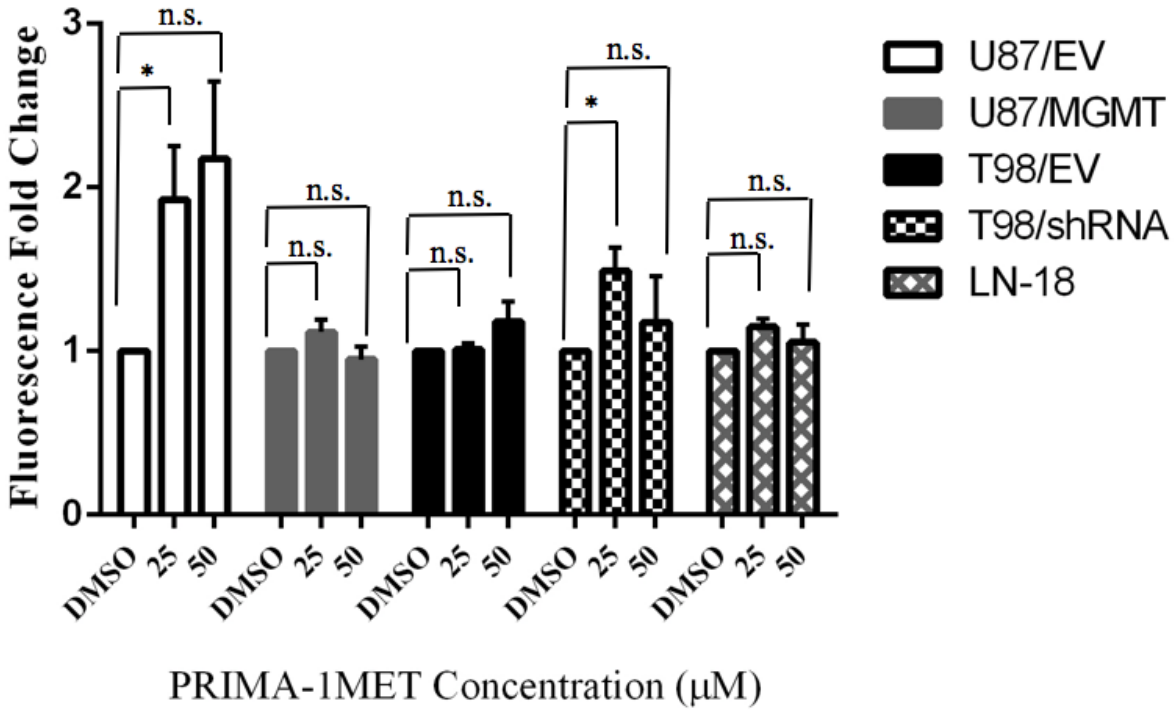
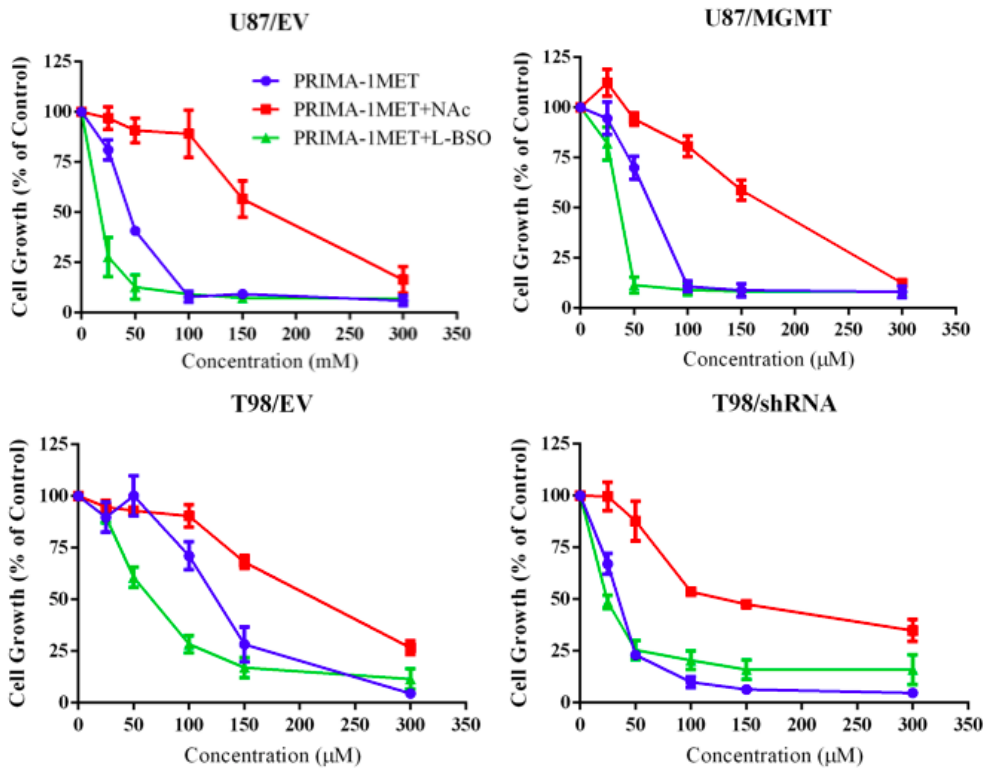


Figure 2.2

A.


U87/EV
U87/MGMT
T98/EV
T98/shRNA
PRIMA-1MET
PRIMA-1MET+NAc
PRIMA-1MET+L-BSO
Cell Growth (% of Control)
Concentration (mM)
Concentration (μM)

B.

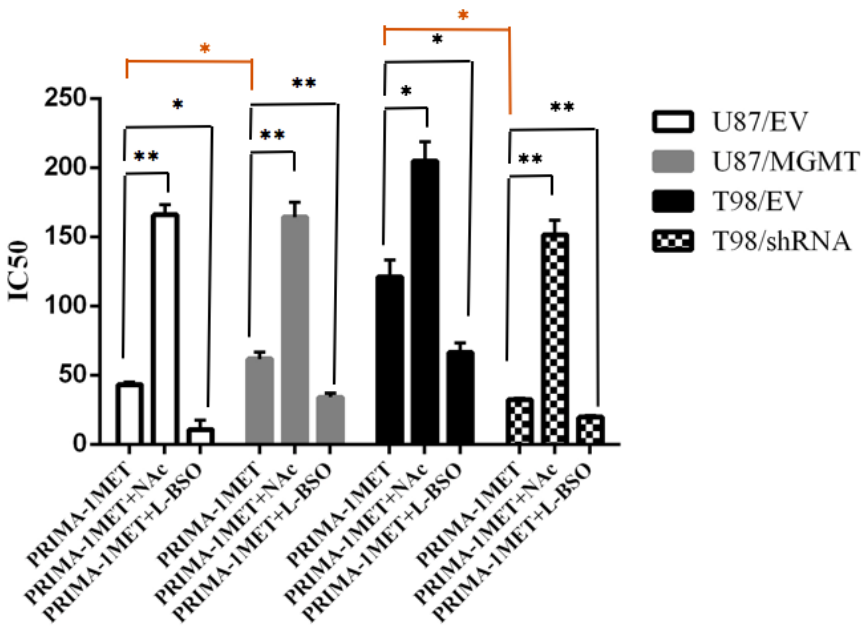

IC50
U87/EV
U87/MGMT
T98/EV
T98/shRNA
PRIMA-1MET
PRIMA-1MET+NAc
PRIMA-1MET+L-BSO

Figure 2.3

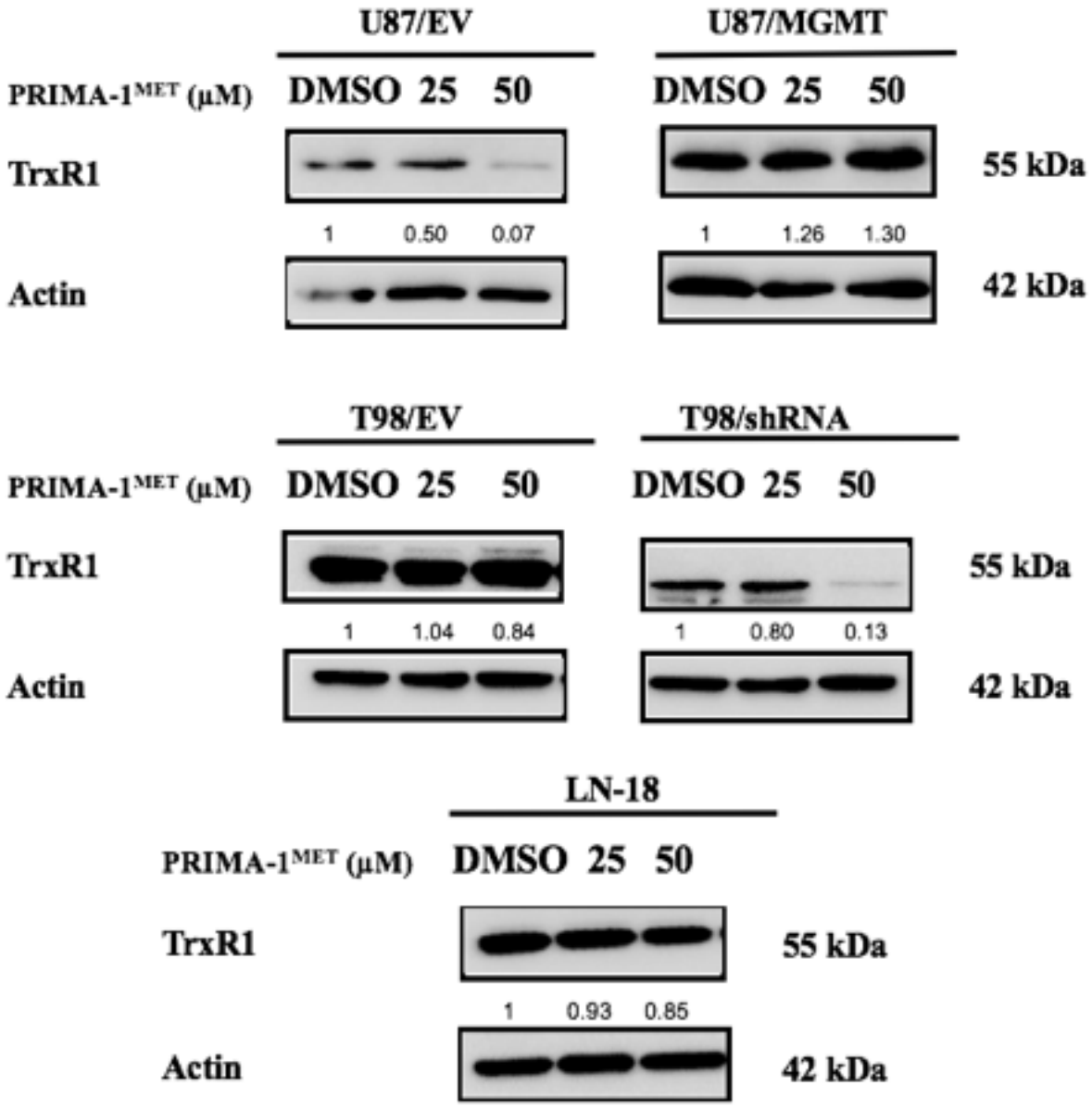

U87/EV
U87/MGMT
PRIMA-1MET (μM)
DMSO 25 50
DMSO 25 50
TrxR1
55 kDa
1 0.50 0.07
1 1.26 1.30
Actin
42 kDa
T98/EV
T98/shRNA
PRIMA-1MET (μM)
DMSO 25 50
DMSO 25 50
TrxR1
55 kDa
1 1.04 0.84
1 0.80 0.13
Actin
42 kDa
LN-18
PRIMA-1MET (μM)
DMSO 25 50
TrxR1
55 kDa
1 0.93 0.85
Actin
42 kDa

Figure 2.4

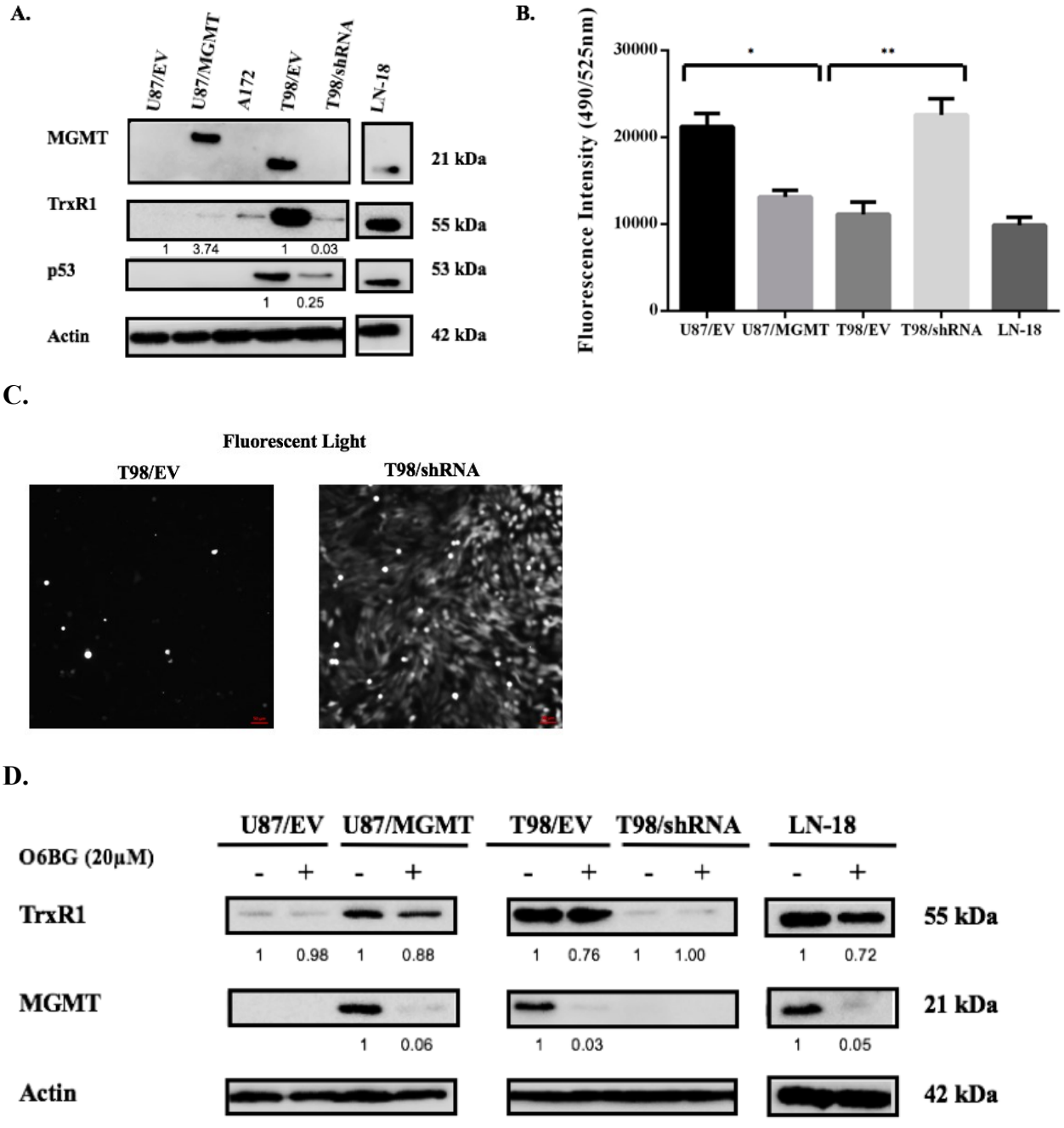
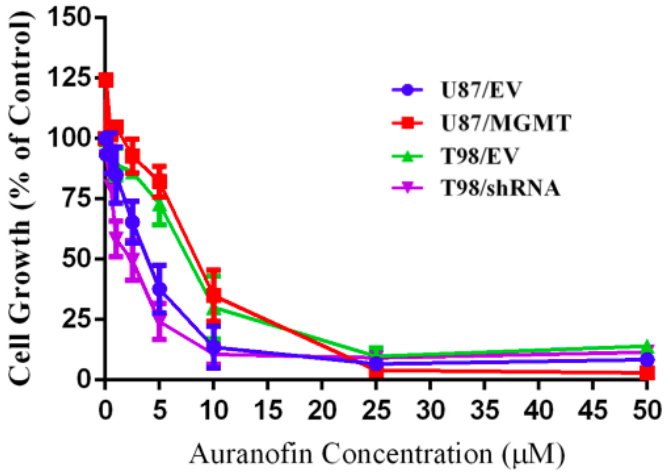

A.
U87/EV
U87/MGMT
A172
T98/EV
T98/shRNA
LN-18
MGMT
TrxR1
p53
Actin
21 kDa
55 kDa
53 kDa
42 kDa
1 3.74 1 0.03
1 0.25
B.
Fluorescence Intensity (490/525nm)
30000
20000
10000
0
*
**
U87/EV U87/MGMT T98/EV T98/shRNA LN-18
C.
Fluorescent Light
T98/EV
T98/shRNA
D.
U87/EV U87/MGMT T98/EV T98/shRNA LN-18
O6BG (20μM)
- + - + - + - + - +
TrxR1
55 kDa
1 0.98 1 0.88 1 0.76 1 1.00 1 0.72
MGMT
21 kDa
1 0.06 1 0.03 1 0.05
Actin
42 kDa

Figure 2.5

A.

A)



Cell Line	IC50 (µM)	p-Value
U87/EV	3.6	0.0311
U87/MGMT	8.3	
T98/EV	7.7	0.0116
T98/shRNA	2.1	

B.

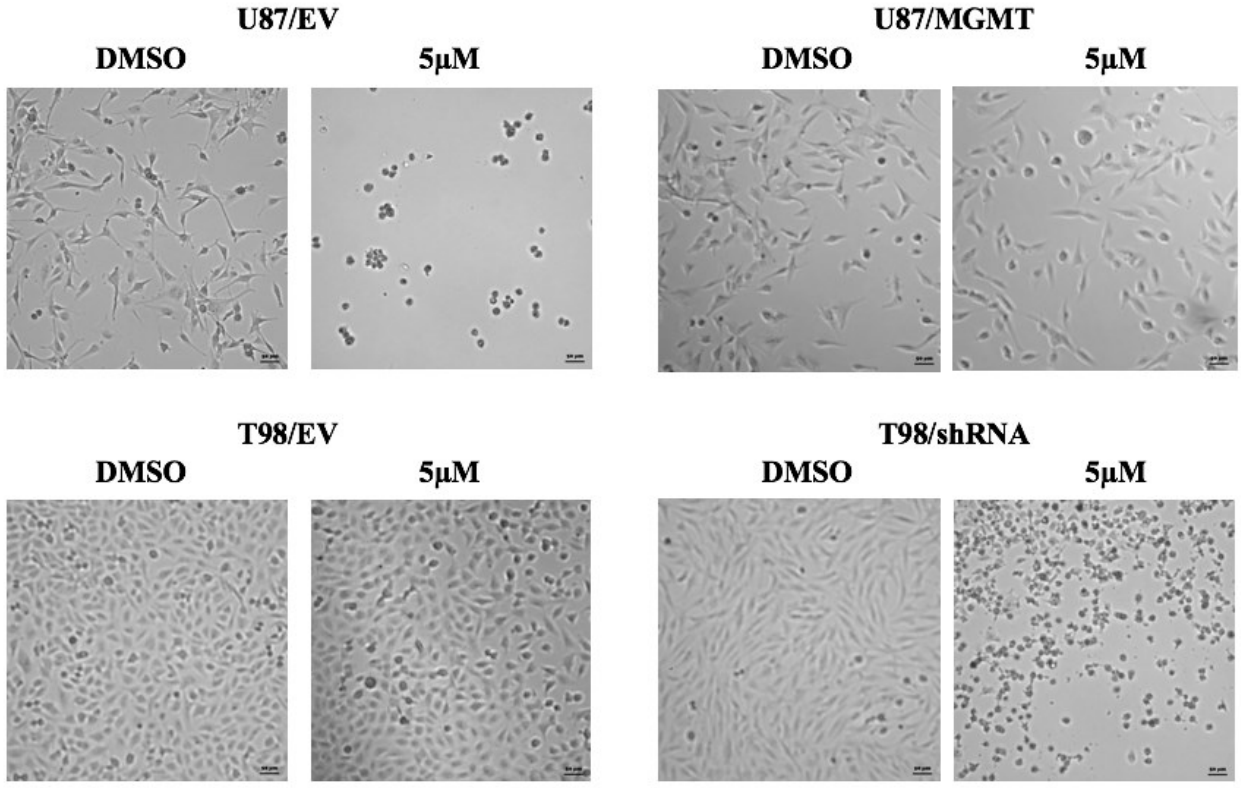
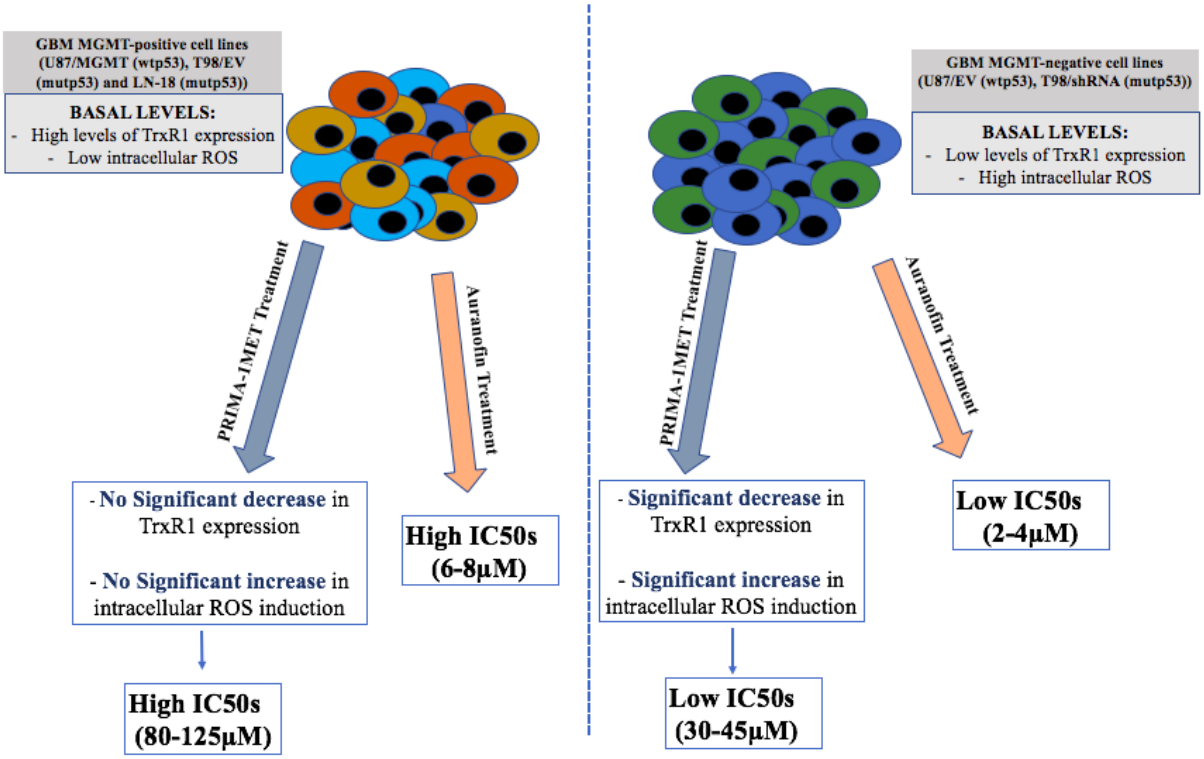


Figure 2.6


GBM MGMT-positive cell lines (U87/MGMT (wtp53), T98/EV (mutp53) and LN-18 (mutp53))
BASAL LEVELS:
- High levels of TrxR1 expression
- Low intracellular ROS
PRIMA-1MET Treatment
Auranofin Treatment
- No Significant decrease in TrxR1 expression
- No Significant increase in intracellular ROS induction
High IC50s (6-8µM)
High IC50s (80-125µM)
GBM MGMT-negative cell lines (U87/EV (wtp53), T98/shRNA (mutp53))
BASAL LEVELS:
- Low levels of TrxR1 expression
- High intracellular ROS
PRIMA-1MET Treatment
Auranofin Treatment
- Significant decrease in TrxR1 expression
- Significant increase in intracellular ROS induction
Low IC50s (2-4µM)
Low IC50s (30-45µM)

2.9 Figure Legends

Figure 2.1. Induction of ROS by PRIMA-1MET in GBM cell lines irrespective of p53 status. (A) Western blotting analysis of basal levels of MGMT and p53 in lysates of U87MG transfected with an empty vector (U87/EV) or with a plasmid carrying exogenous MGMT (U87/MGMT), T98G transfected with empty vector (T98/EV) and shRNA-mediated knockdown of MGMT (T98/shRNA), A172, U138, and LN-18 GBM cell lines. **(B)** U87/EV, U87/MGMT, T98/EV, T98/shRNA and LN-18 GBM cell lines were treated with PRIMA-1MET at concentrations (25μM, and 50μM) for 24 hrs, labelled with 10μM CM-H2DCFDA and ROS levels were quantified by a fluorescent microplate reader. Graphs represent mean values ± SEM from at least four independent experiments performed in triplicate. Student's t-test was performed to measure significance relative to control: *$p<0.05$ and n.s.- non-significant.

Figure 2.2. N-Acetylcysteine inhibits cytotoxicity of PRIMA-1MET in GBM MGMT cell lines whereas, L-Buthionine Sulfoximine enhances its effect. (A)GBM cell lines (U87/EV, U87/MGMT, T98/EV, and T98sh/RNA) were treated with 0-300μM PRIMA-1MET alone or in combination with either NAc (1mM) or L-BSO (10μM) for 72hrs and growth-inhibitory effects were examined by an MTT assay. **(B)** Comparisons of IC50s between each condition. Graphs represent mean values ± SEM from at least three independent experiments performed in triplicate. Student's t-test was performed to measure significance relative to control: *$p<0.05$, **$p<0.01$, *** $p<0.001$, n.s.- non-significant.

Figure 2.3. PRIMA-1MET decreases TrxR1 in cells lines irrespective of p53 status. Western blotting analysis of 24hr PRIMA-1MET treatment at concentrations (25μM, and 50μM) on TrxR1 expression. Expression of TrxR1 in lysates of the following cell lines GBM cell lines: U87/EV, U87/MGMT, T98/EV, T98/shRNA and LN-18. Density of bands with normalized to their DMSO control and actin was used as a loading control.

Figure 2.4. MGMT upregulation is associated with increased TrxR1 protein levels and low intracellular ROS levels and MGMT silencing decreased TrxR1 protein levels and increased intracellular ROS. (A) Western blotting analysis of the effect of MGMT silencing and overexpression on TrxR1 expression. Expression of TrxR1, MGMT, and p53 in lysates of U87/EV, U87/MGMT, A172, T98/EV, T98/shRNA, and LN-18 GBM cell lines. Density of bands were normalized to their respective empty vector. **(B)** Basal levels of ROS in cell lines were quantified by a fluorescent microplate reader after loading cells with 10μM CM-H2DCFDA for 30minutes in GBM MGMT isogenic cell lines and LN-18. Student's t-test was performed to measure significance relative to MGMT isogenic counterpart: *$p<0.05$, and **$p<0.01$. (**C)** Fluorescent micrographs visualized from Zeiss inverted microscope (Zeiss Axio Vert.A1, Germany) show differential levels of ROS via fluorescence in T98G MGMT isogenic cell lines at a magnification of 10x. **(D)** Western blotting analysis of expression of TrxR1 and MGMT in U87/EV, U87/MGMT, T98/EV, T98/shRNA and LN-18 following 48h treatment with O6-Benzylguanine, O6-BG (20 μM). Density of bands were normalized to DMSO control and actin was used as a loading control.

Figure 2.5. Auranofin decreased proliferation of GBM isogenic MGMT cell lines irrespective of p53 status. (A) GBM MGMT isogenic cell lines (U87/EV, U87/MGMT,

T98/EV, and T98sh/RNA) were treated with 0-25μM Auranofin for 72hrs and growth-inhibitory effects were examined by an MTT assay. The resulting IC50 values are shown in the table. Graphs represent mean values ± SEM from at least three independent experiments. **(B)** Representative micrographs of GBM isogenic cell lines at 10X magnification following treatment with 5μM of Auranofin or DMSO control for 48hrs.

Figure 2.6. Model summarizing our findings for the basal redox levels (reactive oxygen species (ROS), and thioredoxin reductase 1 (TrxR1) in MGMT-positive and MGMT-negative cell lines and the effects of PRIMA-1MET and Auranofin.

2.10 Tables

Cell Line	Condition	IC50 (μM)	Fold Change **	p-Value**
U87/EV	PRIMA-1MET	43	1	
	PRIMA-1MET+NAc	166	3.8	0.0024
	PRIMA-1MET+L-BSO	10	0.3	0.0360
U87/MGMT	PRIMA-1MET	84	1	
	PRIMA-1MET+NAc	164	2.0	0.0040
	PRIMA-1MET+L-BSO	34	0.4	0.0137
T98/EV	PRIMA-1MET	121	1	
	PRIMA-1MET+NAc	205	1.7	0.0115
	PRIMA-1MET+L-BSO	66	0.6	0.0260
T98/shRNA	PRIMA-1MET	32	1	
	PRIMA-1MET+NAc	152	4.7	0.0069
	PRIMA-1MET+L-BSO	20	0.6	0.0017

** Fold Change and p-value are calculated compared to the PRIMA-1MET alone IC50 for each respective cell line.

2.11 Table Legend

Table 2.1 IC50 values for the combination of PRIMA-1MET with ROS modulating agents: ROS scavenger, N-acetylcysteine and ROS inducer, L-Buthionine-Sulfoximine.

Chapter 3.
General Discussion and Conclusion

The purpose of this thesis was to investigate the mechanisms underlying previous findings reported by our team (71), specifically the p53-independent effects and MGMT-based differential effects of PRIMA-1MET in the context of GBM. The following section will provide additional discussion and a summary of our findings.

3.1 p53- independent effects of PRIMA-1MET in GBM

PRIMA-1MET/APR-246 is a small molecule originally designed to selectively target mutp53 and restore its wtp53 function (143), however, it was later reported in several cancer models, to be effective in cell lines with either mutp53 or wtp53 background (144). Accordingly, our lab has recently reported PRIMA-1MET p53-independent effects in GBM cell lines and brain tumor initiating (stem) cells (BTICs). Previous studies have proposed various p53-independent mechanisms for PRIMA-1MET including: *(i)* reactivation of inactivated wtp53, *(ii)* modulation of redox balance and *(iii)* activation of the unfolded protein response in the endoplasmic reticulum (144).

Among these mechanisms, the modulation of redox balance specifically through induction of reactive oxygen species (ROS) and inhibition of antioxidants i.e. thioredoxin reductase 1 (TrxR1) and glutathione were frequently reported (153, 154, 210). In the present study, we demonstrated that PRIMA-1MET induces ROS irrespective of p53 status and identified expression of MGMT as a key element in PRIMA-1MET-mediated ROS increase in GBM cell lines isogenic for MGMT. Furthermore, we found that PRIMA-1MET decreased levels of TrxR1 more in MGMT-negative cell lines compared to their counterparts, which correlated well with our findings for the levels of ROS in these cell lines. In the context of PRIMA-1MET, a recent study conducted by Peng et al., demonstrated that the inhibition of TrxR1 by methylene qunuclidinone, and its conversion to a pro-oxidant NADPH oxidase is crucial for PRIMA-1MET mediated induction of ROS in lung adenocarcinoma and osteosarcoma cell lines (210). They further validated this when treatment of cells with downregulation of TrxR1 decreased levels of ROS. Therefore, our results suggest that the induction of ROS following treatment with PRIMA-1MET are likely due to targeting of TrxR1 by PRIMA-1MET. This mechanism would need to be further validated in the context of GBM.

Our results reveal that the PRIMA-1MET synergized well with L-Buthithione-Sulfoximine in GBM. This raises the question of whether the glutathione system may be also

inhibited by PRIMA-1MET as suggested in Tessoulin et al. (154). The role of nuclear factor (erythroid-derived 2)-like2 (Nrf2), a master regulator of antioxidants such as glutathione and thioredoxin needs also to be assessed in this context. Additional limitations of our results include the need to assess the effects of PRIMA-1MET in the levels of ROS in a large panel of GBM cell lines and BTICs. In addition, although we have shown that the generation of ROS is necessary for PRIMA-1MET p53-independent effects, as co-treatment N-Acetylcysteine reversed PRIMA-1MET anti-proliferative effects, we did not investigate the molecular mechanisms by which ROS and inhibition of TrxR1 induce cell death. Moreover, ROS has been implicated in mediating DNA damage and cell death through activation of transmembrane death receptors or pro-apoptotic proteins (191). A study conducted by Saitoh et al., showed that the reduced form of thioredoxin is involved in inhibiting apoptosis signal-regulating kinase (ASK)1, in addition, ROS may oxidize thioredoxin (Trx) and prevent is binding and inhibition of ASK 1 (211). This may be a possible mechanism by which inhibition of TrxR1 induces apoptosis. Inhibition of TrxR1 prevents reduction of Trx, which in turn prevents inhibition of ASK 1. PRIMA-1MET induction of ROS might also prevent inhibition of ASK1. Among the studies that investigated the mechanisms of ROS-induced cell death of PRIMA-1MET, the majority have proposed that it is via activation of caspases (144) while few studies proposed that PRIMA-1MET induce activation of autophagy. One example is the study of the effects of PRIMA-1MET in soft-tissue sarcoma in which PRIMA-1MET led to the activation of JNK pathways which in turn led to autophagy (168). The mechanisms underlying PRIMA-1MET p53-independent mechanisms for DNA damage cell death through modulation of redox signaling need to be further investigated in GBM.

Current anti-cancer therapeutic strategies take advantage of the reliance of cancer cells on redox balance to develop agents that augment intracellular levels of ROS. The fact that we provide evidence for PRIMA-1MET to alter redox balance in GBM cell lines is of clinical significance. This also suggests that we can take advantage of this mechanism to potentially design new combination strategies using PRIMA-1MET with other agents in GBM. Rocha et al., proposed that resistance to TMZ may be associated with low levels of ROS production or glutathione scavenging action for TMZ which may interfere with TMZ cytotoxicity (212). Therefore, PRIMA-1MET may be able to sensitize cells to TMZ by inducing ROS. Other potential treatment paradigms include combining PRIMA-1MET with agents that increase ROS

to complement the cytotoxic activity of PRIMA-1MET such as Auranofin (213), Curcumin (214), etc. Therefore, further understanding its mechanism of action will enable the development of efficient treatment combinations to increase its cytotoxicity in GBM.

3.2 Role of MGMT in PRIMA-1MET resistance

The DNA repair protein, MGMT is an important resistant factor in GBM treatment as it interferes with the cytotoxic effect of TMZ, a major component of conventional GBM treatment (49). In our previous study, high MGMT expression was correlated with increased resistance to PRIMA-1MET (71) prompted the current study to investigate in this thesis the mechanisms underlying the differential growth inhibitory effects of PRIMA-1MET based on expression on expression of MGMT. Our results (Chapter 2) shed light into the p53-independent effects of PRIMA-1MET through inhibition of TrxR1 and ROS and the differential effects based on MGMT expression. PRIMA-1MET decreased TrxR1 significantly and induced ROS at lower concentrations in MGMT-negative cell lines compared to MGMT-positive cell lines. Furthermore, combined treatment of these cells with PRIMA-1MET and N-Acetylcysteine, compared to PRIMA-1MET alone reduced MGMT-based differences (Figure 2.2A, Table 2.1) suggesting the role of MGMT in modulating redox balance in GBM cell lines. We also demonstrated that GBM cell lines constitutively express differential basal levels of TrxR1 and ROS such that cells with high MGMT expression, have high TrxR1 expression and low ROS levels and vice versa (Figure 2.4). This therefore, might account for the differential effects of PRIMA-1MET which exerts its effects as a TrxR1 inhibitor in GBM as these cells naturally display different levels of its target TrxR1. Furthermore, the differential levels of TrxR1 as a target was further validated by the differential effects based on MGMT expression levels of the TrxR1-targeting drug, Auranofin, though with more drastic effects compared to PRIMA-1MET in these cell lines (Figure 2.5).

In this research project, we propose a novel relationship between MGMT and TrxR1 to explain MGMT-mediated resistance of PRIMA-1MET in MGMT-positive cell lines. This potential relationship sheds light into the multifaceted role of MGMT in GBM and a better understanding of PRIMA-1MET mechanism to develop better treatment strategies to improve survival of patients diagnosed with GBM.

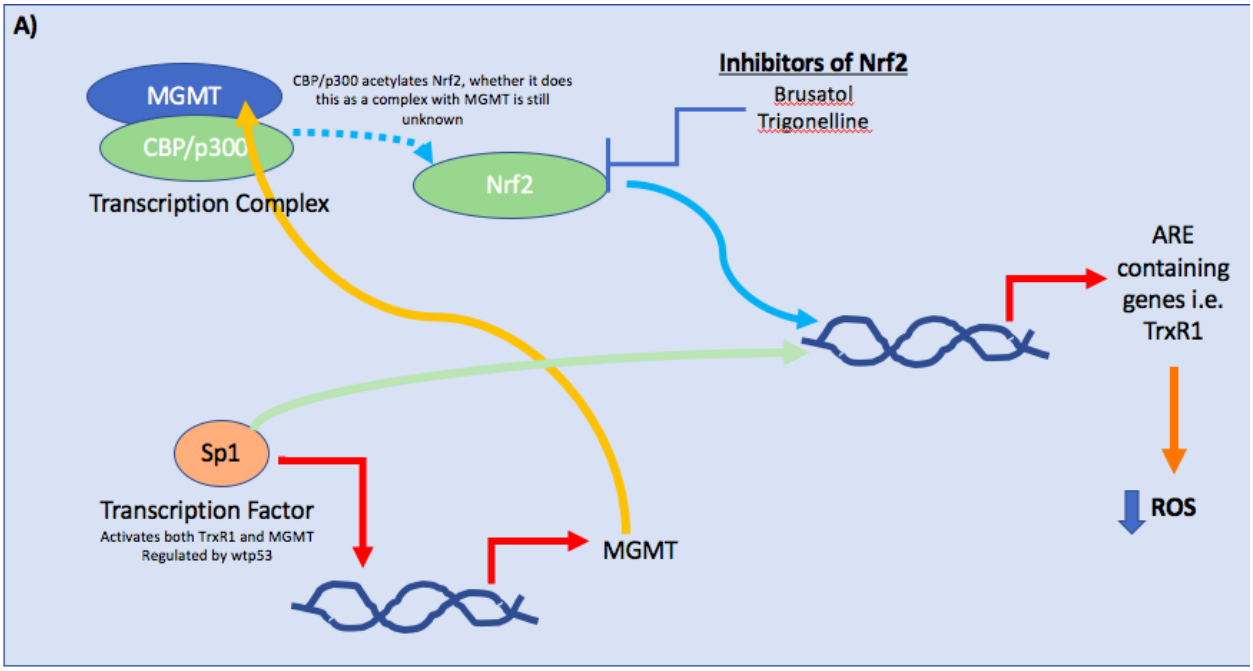
The possible mechanisms to explain the positive correlation between MGMT levels and

increased expression of TrxR1 could be related to the potential following mechanisms: *(i)* MGMT binds directly to the *TXNRD1* gene promoter and enhances its expression, *(ii)* both MGMT and TrxR1 are activated by a common transcription factor, or *(iii)* MGMT regulates expression of TrxR1 indirectly via an intermediate factor. In a previous study, MGMT has been suggested to function as an ancillary protein that forms a complex with the CREB-binding protein, CBP/p300 and is therefore, involved in regulating expression of various genes (56). Interestingly, CBP/p300 acetylates Nrf2, a key regulator of antioxidant pathways in the cell and defense mechanism against oxidative stress, which in turn activates transcription of antioxidant response element (ARE) containing genes such as i.e. TrxR1 (Figure 3.1A) (102). Additional experiments to investigate this relationship include assessing the localization of MGMT, Nrf2 and TrxR1, studying the effects of the molecular expression of Nrf2 and TrxR1 following knockdown of MGMT or pharmacological inhibition of Nrf2 by Brusatol (215-217) and Trigonelline (218, 219).

Sharing a common transcription factor is another potential explanation for the relationship between MGMT and TrxR1. One notable candidate is specificity protein 1 (Sp1), which is an important transcription factor for both genes (65, 220). Sp1 may activate transcription of *MGMT* and therefore lead to the activation of TrxR1 indirectly or Sp1 may bind directly to the *TXNRD1* gene promoter and activate it (Figure 3.1A). Levels of Sp1 can be regulated by wildtype p53 which has been associated with sequestering Sp1 and therefore, inhibiting expression of MGMT. In addition, it has been demonstrated that Sp1 overexpression can revert p53-mediated inhibition of MGMT (65), which showcases the importance of Sp1 in the relationship between MGMT and p53. Whether p53 is involved in the cross talk between MGMT and TrxR1 requires additional investigation.

Transcription of *NFE2L2* gene encoding Nrf2 has been previously correlated with TrxR1 upregulation (201). More specifically, in response to oxidative stress, NRF2 accumulates and translocates to the nucleus and activates transcription of ARE containing genes i.e. TrxR1 in a mutp53-dependent mechanism (221). Mutp53 can upregulate Nrf2-mediated activation of ARE containing genes such as TrxR1 by binding to Nrf2 and sequestering it into the nucleus to activate Nrf2 regulatory elements (221). Lisek et al. showed that Nrf2 silencing abolished mutp53-dependent increase of TrxR1 mRNA levels (221). Our results corroborate these findings, as mutp53/MGMT-positive cell lines had higher expression of TrxR1 compared to the

wtp53/MGMT-positive cell line. Interestingly, in a previous study we found a decrease in mutp53 following MGMT silencing in a T98G GBM model with a mutp53 background (71). This relationship could potentially explain the decreased TrxR1 expression levels in T98/shRNA cell line knockdown for MGMT. Decrease in mutp53 by MGMT can reduce Nrf2 activation of ARE-containing genes i.e. TrxR1 and therefore, reduce its expression (Figure 3.1B). Both these models do not exclude the potential role of MGMT in the differential expression of other antioxidant enzymes such as in the glutathione system, which could be investigated in future studies.



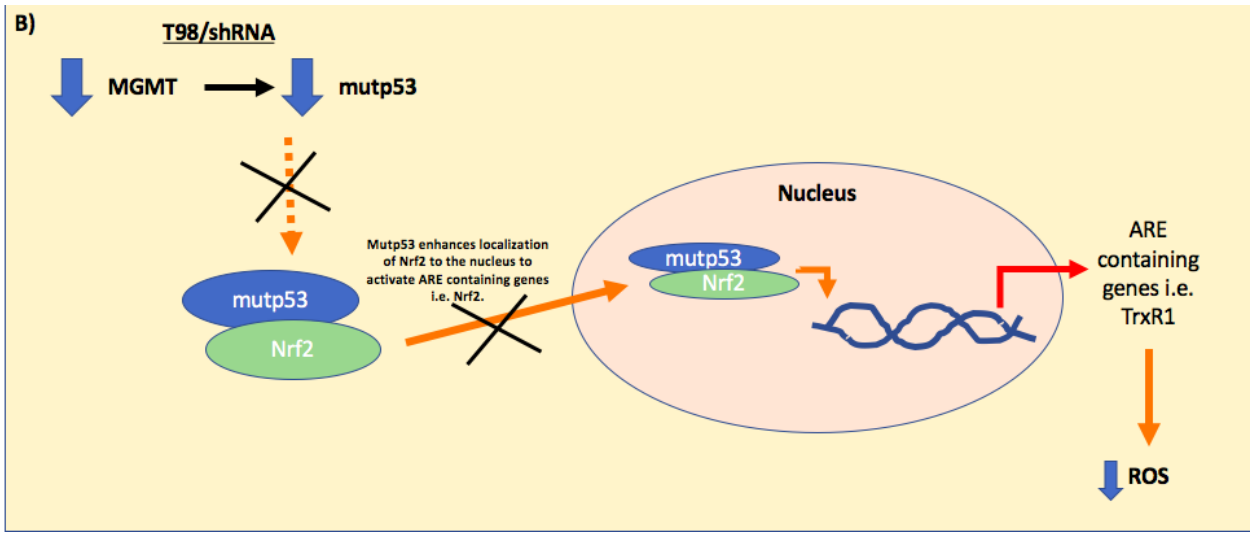


Figure 3.1. Hypothetical model representing the potential mechanisms underlying the relationship between MGMT, TrxR1 and ROS. (A) A potential mechanism independent of p53 status: Sp1, a transcription factor regulated by wtp53 activates transcription of *MGMT* gene. MGMT forms a complex with CREB-binding protein, CBP/p300 to acetylate nuclear factor erythroid 2-related factor 2, Nrf2 leading to activation of downstream genes that contain antioxidant response elements such as thioredoxin reductase 1 (TrxR1). TrxR1 is an important regulator of reactive oxygen species (ROS). Sp1 also binds to the promoter of TrxR1 and activate it**. (B)** A mutp53 dependent mechanism: In T98/shRNA, we saw that MGMT silencing reduced expression of mutp53, which prevents mutp53-dependent nuclear translocation of Nrf2 to activate ARE-containing genes. Solid lines represent the relationships experimentally investigated, whereas dotted lines represent the relationships that need to be further investigated.

The relationship between MGMT and TrxR1 may concomitantly provide an explanation of the differences in levels of ROS. This is because as mentioned in Chapter 1, TrxR1 is an important protein involved in thioredoxin regulation of ROS (198). A study conducted by Hwang-Bo et al., demonstrated that Auranofin-mediated inhibition of TrxR1 induced ROS in hepatocellular carcinoma (222) .

Several studies have proposed the role TrxR1 in many important cellular functions notably in cell proliferation, regulation of redox balance, angiogenesis, alterations in morphology, and tumorigenesis (199). The potential link between MGMT and redox balance may possibly explain certain properties we observed previously when comparing MGMT isogenic cell lines, such as alterations in the morphology of T98G cell lines as they became more elongated following MGMT knockdown (71). This could be potentially related to changes in TrxR1 expression levels in these cell lines. Moreover, previously we reported an anti-angiogenic profile in MGMT-positive wtp53 cell lines with high vascular endothelial growth factor receptor 1 (VEGFR-1)/VEGFA ratio (57). In a study conducted by Streicher et al., they found that chemical inhibition of TrxR1 was associated with increased expression of VEGF and VEGF receptor expression and angiogenesis (223). This is consistent with previous findings from our group showing an anti-angiogenic profile in U87/MGMT cell line, which has been shown to express high levels of TrxR1 in the current study. The relationship between MGMT and TrxR1 is of potential clinical relevance as TrxR1 levels can be assessed in the blood (205), it might represent a readout for MGMT status in patients diagnosed with GBM.

Although this study is the first to propose a relationship between MGMT and redox balance, there are certain limitations. Further investigation is needed to conclude this association i.e. study of a larger panel of GBM cell lines, in silico analysis of the relationship between

MGMT and TrxR1 and other antioxidants i.e. glutathione or Nrf2 using publicly available cell line databases and patient genomic analysis, validation in brain tumor stem cells (BTSCs), etc.

Taken together, our results provide the first evidence that the differential effects of PRIMA-1MET based on MGMT status are related to differences in basal levels of ROS and TrxR1 in GBM. In addition, it provides preliminary insights into the potential relationship between MGMT and redox balance, which might account for the differential effects of PRIMA-1MET based on MGMT status.

3.3 Summary

In a previous study, our lab reported the cytotoxic effects of PRIMA-1MET in BTICs (71) known for their role as essential drivers of tumor initiation and for their resistance to chemoradiation. However, cells with high MGMT expression were found to be resistant irrespective of p53 status (71), suggesting that PRIMA-1MET might not be further developed as a promising agent in MGMT-positive GBM. Therefore, the main goal of the current study for my Master's thesis was to investigate the mechanisms underlying resistance of MGMT-positive cells to PRIMA-1MET and its p53-independent effects in GBM.

Our results reveal that PRIMA-1MET mediates its cytotoxic effect irrespective of p53 status by decreasing levels of thioredoxin reductase 1(TrxR1) and induction of reactive oxygen species (ROS). Using isogenic GBM cell lines for MGMT, we showed that MGMT-based differences in PRIMA-1MET cytotoxicity was based on differential basal levels of ROS and TrxR1. In addition, we demonstrated that beyond its known function to restore wtp53 function to mutp53, PRIMA-1MET exerts its cytotoxic effects as an inhibitor of TrxR1 in GBM. Therefore, a higher concentration of PRIMA-1MET is required to decrease levels of TrxR1 in MGMT-positive cells, ultimately explaining the differential growth inhibitory effects of PRIMA-1MET in MGMT isogenic cell lines observed in our previous study. This relationship was further validated when a known irreversible TrxR1 inhibitor, FDA-approved drug, Auranofin, also showed differential effects in these isogenic cell lines.

Furthermore, previous studies have primarily focussed on studying the effects of PRIMA-1MET in the context of mutp53 or wtp53 cellular background. Our results thus, provide the first evidence in support of our previous findings of PRIMA-1MET in GBM for the importance of MGMT expression and shed light for future studies examining the efficacy of p53-targeting

agents to do so in the context of MGMT, redox balance or other potential proteins involved in this cross-talk.

Through investigation of the effects of PRIMA-1MET in both mutp53 and wtp53 cell lines isogenic for MGMT, we identified an intriguing, novel relationship between MGMT and redox balance specifically the expression of TrxR1 and levels of basal ROS. We are the first lab to assess this association. Thus, these findings in our present study are relatively new and examined in only in a small panel of cells and limited methodology, therefore, we were not able to investigate the mechanisms underlying this association. In this thesis, we also discussed hypothetical mechanisms to explain this association, and therefore, anticipate that our findings will lay the foundation for future experiments ultimately leading to the potential clinical use of TrxR1 targeting drugs such as PRIMA-1MET and Auranofin in GBM treatment.

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