Interplay between O6-Methylguanine-DNA Methyltransferase (MGMT) and p53 in Glioblastoma: Implications for Response to p53-targeting Compound PRIMA-1^{MET} (APR-246) and Ionizing Radiation

by

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August 2016

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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ABSTRACT

Glioblastoma multiforme (GBM) is the most frequent and most aggressive form of malignant brain tumor in adults with a median survival time of only 15 months despite treatment with surgery, concurrent radiation therapy (RT) and temozolomide (TMZ)-based chemotherapy. The DNA repair protein, O6-methylguanine-DNA-methyltransferase (MGMT) counteracts TMZ-induced DNA alkylation and mediates chemoresistance. Alterations of the *TP53* tumor suppressor gene occur in ~30% of primary GBM with a high frequency of missense mutations associated with the acquisition of oncogenic "gain-of-function" (GOF) mutant (mut)p53 activities. New rational therapeutic strategies are urgently needed to implement effective targeted therapies and circumvent chemoradioresistance.

In this PhD thesis, we focused on the interplay between MGMT and p53, key determinants of GBM chemoradioresistance, and how *TP53* status and expression of MGMT may affect response to PRIMA-1^{MET}/APR-246, a small molecule designed to rescue wild-type (wt)p53 function, either as a single agent or in combination with ionizing radiation (IR). We showed that MGMT silencing decreased expression of GOF/mutp53 protein. Using a GOF/mutp53 GBM cell line silenced for MGMT, we showed that PRIMA-1^{MET} further decreased expression of mutp53, decreased proliferation and clonogenic potential, abrogated the G_2 checkpoint control, increased susceptibility to apoptotic cell death, expression of GADD45A and sustained expression with cytosolic localization of phosphorylated Erk1/2 kinases. Interestingly, PRIMA-1^{MET} decreased relative cell number, disrupted the structure of neurospheres of patient-derived GBM stem cells (GSCs) and either enabled activation of wtp53 with decreased expression of MGMT in MGMT-positive GSCs or decreased expression of mutp53 protein in a mutp53 GSC line.

Further investigation of the effect of PRIMA-1^{MET} combined to IR showed that PRIMA-1^{MET} is capable of causing DNA damage in MGMT low / mutp53 GBM cell lines and highlighted

the radiosensitizing effects of PRIMA-1^{MET} based on expression of MGMT in mutp53 GBM cells. While the effects of PRIMA-1^{MET} are independent of restoring wtp53 functions, we identified different mechanisms of cytotoxicity and cell fate leading to senescence or apoptosis in GBM cells expressing low levels of MGMT and harboring wild-type or mutp53 protein, respectively.

In sum, our study suggests a reciprocal relationship between MGMT and p53 in GBM, which in turn affects response to PRIMA-1^{MET}. Our results further highlight the cell-context dependent effects of PRIMA-1^{MET} alone and in combination with IR irrespective of p53 status and provide the basis for investigating these effects using *in vivo* GBM orthotopic models. These studies will provide the proof-of-principle for the use of PRIMA-1^{MET} alone or in combination with TMZ and/or IR as a novel therapeutic approach for GBM tumors unresponsive to standard treatment based on *TP53* status and expression of MGMT.

RÉSUMÉ

Le glioblastome multiforme (GBM) est la forme la plus fréquente et la plus agressive de tumeur malignes du cerveau chez les adultes avec une durée médiane de survie de seulement 15 mois malgré un traitement par la chirurgie, la radiothérapie concurrente (RT), et la chimiothérapie à base de témozolomide (TMZ). La protéine de réparation d'ADN, O6-méthylguanine-ADN-méthyltransférase (MGMT) contourne les effets de l'alkylation de l'ADN induite par TMZ et favorise la chimiorésistance. Les altérations du gène suppresseur de tumeur *TP53* se produisent dans environ 30% du GBM primaire avec une fréquence élevée de mutations faux-sens associées à l'acquisition d'activités oncogènes de "gain de fonction" (GOF) du mutant (mut) p53. De nouvelles stratégies thérapeutiques rationnelles sont nécessaires de toute urgence pour mettre en œuvre des thérapies ciblées efficaces et contourner la chimioradiorésistance. Dans cette thèse, nous nous sommes concentrés sur la relation entre MGMT et p53, les déterminants clés de la chimioradiorésistance du GBM, et comment le statut de *TP53* et l'expression de MGMT peut affecter la réponse à PRIMA-1^{MET} / APR-246, une petite molécule destinée à restaurer la fonction de wtp53, soit en tant que monothérapie ou en association avec la radiothérapie ionisante (IR). Nous avons montré que la répression de l'expression de MGMT entraine une diminution de l'expression de la protéine GOF/mutp53. L'utilisation d'une lignée cellulaire de GBM avec GOF/mutp53 et répression de MGMT, nous avons montré que PRIMA-1^{MET} diminue davantage l'expression de mutp53, diminue la prolifération et le potentiel clonogénique, inhibe le point de contrôle du G2, augmente la sensibilité à la mort cellulaire apoptotique, l'expression de Gadd45a, et maintient l'expression avec localisation cytosolique des ERK1/2-kinases phosphorylées. Il est intéressant de noter que PRIMA-1^{MET} a diminué le nombre relatif de cellules, a perturbé la structure de neurosphères de cellules souches de glioblastome provenant de patients (GSC) et a entrainé soit l'activation de wtp53 avec une diminution de l'expression de MGMT dans les GSCs

MGMT-positives, ou la diminution de l'expression de la protéine mutp53 dans une lignée GSC mutp53.

Une étude plus poussée de l'effet de PRIMA-1^{MET} combiné à l'IR a montré que PRIMA-1^{MET} est capable de causer des dommages à l'ADN dans des lignées cellulaires de GBM « MGMT faible expression / mutp53 », et mis en évidence les effets radiosensibilisants de PRIMA-1^{MET} basés sur l'expression de MGMT dans les cellules du GBM mutp53. Alors que les effets de PRIMA-1^{MET} sont indépendants de la restauration des fonctions de wtp53, nous avons identifié différents mécanismes de cytotoxicité et le devenir des cellules conduisant à la sénescence ou l'apoptose dans les cellules de GBM exprimant de faibles niveaux de MGMT et le statut de p53 non-muté ou muté, respectivement.

En somme, notre étude suggère une relation réciproque entre MGMT et p53 dans le GBM, qui à son tour affecte la réponse à PRIMA-1^{MET}. Nos résultats mettent en évidence les effets dépendants des contextes cellulaires de PRIMA-1^{MET} seul et en combinaison avec IR indépendamment du statut de p53 et constituent la base de l'étude de ces effets à l'aide de modèles orthotopiques de GBM in vivo. Ces études fourniront la preuve de principe pour l'utilisation de PRIMA-1^{MET} seul ou en combinaison avec TMZ et / ou IR comme une nouvelle approche thérapeutique pour les tumeurs GBM qui ne répondent pas au traitement standard basé sur le statut de *TP53* et l'expression de MGMT.

ACKNOWLEDGEMENTS

First, I would like to thank my supervisor Dr. Siham Sabri for giving me, as an international student in Canada, an opportunity to join her laboratory and for providing financial support throughout four years of my PhD studies. I would also like to express my sincere gratitude for her continuous support, patience, motivation, enthusiasm and thoughtful guidance. Throughout the course of my PhD studies, Dr. Sabri dedicated a lot of her time to discuss the design of experiments, the interpretation of the obtained results, the ways to resolve technical issues, revise manuscripts, prepare talks, etc., which helped me in all the time of research and writing of thesis.

Also I would like to thank the members of my thesis committee, who guided me through all these years: my academic advisor Dr. Jacques Lapointe, Dr. Bassam Abdulkarim, Dr. Bertrand Jean-Claude and Dr. Jose Teodoro for their insightful comments and encouragement, and for challenging questions, which provided me with an incentive to expand my knowledge.

I am grateful to the McGill-CIHR Drug Development Training Program (DDTP) and Maysie MacSporran graduate studentship, McGill University for financial assistance during my PhD. I also benefited from the financial support provided by Dr. Gerald B. Rice Memorial Travel Award and Great Travel Awards (Division of Experimental Medicine, McGill University) and the McGill-CIHR Drug Development Training Program (DDTP) Travel Awards to present the results of my research at several conferences in Canada and USA.

I extend my sincere word of thanks to the Division of Experimental Medicine, namely Student Affairs Coordinator Mrs. Marylin Linhares, the Student Affairs Officer Mrs. Dominique Besso and the Director Dr. Anne-Marie Lauzon for their help and guidance.

I thank my fellow labmates: Dr. Ayman Oweida, Zeinab Sharifi, Kenny Chatoor, Andrew Lingard, Dr. Nicolas Stifani, Alexandra Candib, Emma Preston, Maryyam Azam, Areej Al Rabea, Bassem Toeama, Dr. Hend Salem, Dr. Gloria Assaker and Dr. Paul Daniel, for the stimulating discussions, exchanges of knowledge and skills, for moral support and for all the fun we have had in the last four years. I thank Di Xue for friendship, nice chats and coffee "therapy" sessions. A sincere thanks also goes to Brian Meehan for cheering me up and "you-can-do-it" greetings.

Finally, this work would not be possible without the tremendous support of my family in Ukraine: my parents, my beloved husband, my sister and her kids - words cannot express how grateful I am for them supporting me spiritually throughout my PhD studies and my life in general.

PREFACE

This thesis is presented in accordance with the manuscript-based thesis guidelines. It contains a submitted peer-reviewed original research article and a research article in preparation for submission. The thesis consists of an introduction (Chapter 1), results (Chapter 2 and 3), discussion (Chapter 4) and appendices. Each research chapter contains sections covering an abstract, introduction, material and methods, results, and discussion as well as its own reference section. Chapter 1 provides a literature review of glioblastoma multiforme (GBM), its molecular subtypes, the problem of tumor heterogeneity and the role of cancer stem cells with an emphasis on the role of MGMT and p53 proteins in GBM chemo- and radioresistance as well as the strategies of reactivation of wild-type p53 functions. Chapter 2 reports the cell-context dependent effects of PRIMA-1^{MET} compound in GBM cells, which depend on MGMT protein levels and additional cell type-specific factors irrespective of p53 status. Chapter 3 investigates the potency of PRIMA-1^{MET} as a radiosensitizing agent in GBM cells with different MGMT expression and p53 status and provides the first evidence that PRIMA-1^{MET} is capable of inducing DNA damage in mutant p53 / MGMT low GBM cells. The thesis has been organized and written by myself. The contributions of myself and of the co-authors are described in the following section.

Contribution to knowledge:

- Manuscript "Sensitivity to PRIMA-1^{MET} is associated with decreased MGMT in human glioblastoma cells and glioblastoma stem cells irrespective of p53 status" was recently published in the journal Oncotarget [1].
- Manuscript "*In vitro* radiosensitization of glioblastoma cell lines by PRIMA-1^{MET} (APR-246): cell-context dependent effects based on expression of MGMT and p53 status" is in preparation for submission to the journal BMC Cancer.

CONTRIBUTION OF AUTHORS

Unless otherwise stated below, I performed experiments, designed and coordinated the experiments not directly conducted by myself, performed analysis of results and wrote the manuscripts that constitute this thesis with guidance from my supervisor Dr. Siham Sabri.

Chapter 2 is reproduced from my manuscript "Sensitivity to PRIMA-1^{MET} is associated with decreased MGMT in human glioblastoma cells and glioblastoma stem cells irrespective of p53 status", recently published in the journal Oncotarget. In this chapter, Ms. Zeinab Sharifi performed treatment of glioblastoma stem cell (GSC) lines with PRIMA-1^{MET}, automated cell counting (for Fig. 2.9B and Fig. 2.11), acquisition of photographs (Fig. 2.10) and preparation of lysates for Western blotting (for Fig. 2.9A and Fig. 2.12). Dr. Kevin Petrecca provided GSC lines. Dr. Jose Mansure assisted with statistical analysis of data and the representation of results of statistical analysis (Fig. 2.1 and Fig. 2.4B – correlation graph). Dr. Bertrand Jean-Claude helped to revise the manuscript.

Chapter 3 is reproduced from my manuscript "*In vitro* radiosensitization of glioblastoma cell lines by PRIMA-1^{MET} (APR-246): cell-context dependent effects based on expression of MGMT and p53 status", which is in preparation for submission to the journal BMC Cancer.

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LIST OF ABBREVIATIONS

AA – anaplastic astrocytoma

Adp53 – adenoviral p53

AGT – O6-alkylguanine DNA alkyltransferase

ATM – ataxia telangiectasia mutated

ATR – ataxia telangiectasia related

BBB – blood-brain barrier

BDP – benzodiazepines

BER – base excision repair

BTICs – brain tumor initiating (stem) cells

BTSCs – brain tumor stem cells

CCLE – Cancer Cell Line Encyclopedia

CI – combination index

CNS – central nervous system

CSCs – cancer stem cells

DAPK – death associated protein kinase

DCs – dendritic cells

DDR – DNA-damage response

DMEM – Dulbecco's modified Eagle's medium

DN – dominant negative

DNMT1 – DNA-methyltransferase 1

DR5 – death receptor 5

DSBs – DNA double strand breaks

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EORTC – European Organization for Research and Treatment of Cancer

ER – endoplasmic reticulum

Erk1/2 – signal-regulated kinases ½

ES – Ewing's sarcoma

FBS – fetal bovine serum

FGF – fibroblast growth factor

GBM – glioblastoma multiforme

G-CIMP – CpG island methylator phenotype

GOF – gain-of-function

GRE – glucocorticoid responsive elements

GSCs – glioma/GBM stem cells

GSH – glutathione

HDAC – histone deacetylases

HIF – hypoxia inducible factor

HRR – homologous recombination repair

HSP – heat-shock protein

IDH1- isocitrate dehydrogenase 1

IFN-β – interferon-β

IHC – immunohistochemistry

IR – ionizing radiation

IRB – Institutional Review Board

JNK – c-Jun N-terminal protein kinase

MAPK – mitogen-activated protein kinase

MGMT – O6-methylguanine DNA methyltransferase

MIRA-1 – mutant p53-dependent induction of rapid apoptosis

miRs – microRNAs

MMR – mismatch repair

MQ – methylene quinuclidinone

MTT – 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

mutp53 – mutant p53

n.s. – not significant

NCIC – National Cancer Institute of Canada

NF-κB – nuclear factor κB

NHEJ – nonhomologous end-joining

NSC – neural stem cells

NSCLC – non-small cell lung cancer

O6-BG – O6-benzylguanine

O6-MeG – O6-methylguanine

OS – overall survival

PARP – poly(ADP-ribose) polymerase

PBMC – peripheral blood mononuclear cells

PBS – phosphate-buffered saline

PDGFRA – platelet-derived growth factor receptor-α

PF – 5-fluorouracil

PFS – progression-free survival

PI – propidium iodide

PI3K – phosphatidylinositol-3 kinase

PRIMA-1 – p53 reactivation and induction of massive apoptosis

PTMs – post-translational modifications

RETRA – reactivation of transcriptional reporter activity

RITA – reactivation of p53 and induction of tumor cell apoptosis

ROS – reactive oxygen species

RPLA – reverse-phase protein lysate microarray

RT – radiotherapy

RTK – receptor tyrosine kinase

SAHA – suberoylanilide hydroxamic acid

SA-β-Gal – senescence-associated beta-galactosidase

SD – standard deviation

SDS – sodium dodecyl sulfate

SER – sensitizer enhancement ratio

SF2 – surviving fraction after 2 Gy

shRNA – short-hairpin RNA

SNP – single nucleotide polymorphism

SSBs – DNA single-strand breaks

STIMA-1 – SH group-targeting compound that induces massive apoptosis

TAD – transcriptional activation domain

TCGA – The Cancer Genome Atlas

TF – transcription factor

TMZ – Temozolomide

TrxR1 – Thioredoxin reductase 1

UTR – untranslated region

VEGF – vascular endothelial growth factor

WHO – World Health Organization

wtp53 – wild-type p53

Chapter 1.

LITERATURE REVIEW & INTRODUCTION

1.1. GLIOBLASTOMA MULTIFORME

Glioblastoma multiforme (GBM), the most malignant and common form of gliomas accounts for approximately 45-50% of all primary brain tumors in adults. GBM is classified by the World Health Organization (WHO) as grade IV [2] astrocytoma that arise from astrocytes, the most abundant glial cells in the brain, closely associated with neuronal synapses and mediating important supportive functions of the brain tissue [3]. GBM is characterized by several histological features including high cellularity, mitotic activity and variety of cell shapes and sizes within the same tumor along with increased angiogenesis and necrosis [4]. GBM is subdivided into *a) GBM, IDH-wildtype*, which corresponds to the so-called primary or *de novo* GBM (80-95% of cases) – with rapid development over about 4 months and the mean age of patients of around 62 years, and *b) GBM, IDH-mutant* corresponding to secondary GBM (~10% of cases) – with evidence of progression from lower grade (II, III) gliomas over the period longer than 6 months (mean length of clinical history ~15 months) typically occurring in patients younger than 45 years [2, 5-7]. Primary and secondary GBM are mostly undistinguishable histologically [8], but possess different genetic and epigenetic characteristics (described below). Despite the aggressive treatment (described below), prognosis for patients diagnosed with GBM remains extremely poor with a median survival of only about 14.6 months [9] due to a number of factors, including tumor invasiveness, high rates of recurrence as well as resistance to radio- and chemotherapy [10].

1.1.1 GBM epidemiology and risk factors

GBM was the most common among malignant primary brain and CNS tumors (45.6%) and accounted for the majority of gliomas (54.7%) in United States in 2007-2011 [11]. The incidence rate for GBM was 3.19 per 100,000 over the period of 2007-2011 or 6.95 per 100,000 annually for adults (40 years or older) and was higher in males (3.98 per 100,000) than in females (2.52 per

100,000) resulting in incidence rate ratio (male:female) of 1.6. GBM is mostly diagnosed in adults older than 35 years and the incidence increases with age (highest rates in individuals 75-84 years old), while a median age of patients is 64 years. The 2-year survival was detected in about 14.8% of cases, while only 5% of patients survived five years post diagnosis during the analyzed period. The survival rate over the 10-year period post-diagnosis negatively correlated with the age of the patients and was the highest for the small number of patients who were diagnosed under age 20 (relative survival rate = 12.6%), followed by a group of patients 20–44 years old at the time of diagnosis (relative survival rate = 10%).

In Canada 85 new cases of GBM and anaplastic astrocytoma have been reported for males and 65 for females in the group of adolescents and young adults (15–29 years) over the period of 2006-2010, while the annual incidence rate was 5.2 per million for males and 4.1 for females [12].

The etiology of GBM remains largely unknown, despite multiple studies trying to link GBM incidence to various risk factors. One of them – exposure to the therapeutic ionizing radiation (IR) or that resulting from atomic bombing was shown to be consistently associated with increased risk of brain tumor development. However the population of individuals that experience such exposure is very low, therefore ionizing radiation could be responsible only for a very small fraction of GBM. A number of hereditary syndromes and diseases was also reported to be associated with brain tumor formation: neurofibromatosis type 1 and 2, tuberous sclerosis, Li-Fraumeni syndrome and Turcot's syndrome [13, 14]. The factors under investigation include electromagnetic radiation, the long-term cell phone use [15-17], cigarette smoking [18, 19] and alcohol consumption [20] as well as allergies and atopic diseases [21].

1.1.2 Standard treatment

Standard of care for GBM patients includes surgical resection of tumor followed by radiotherapy (RT) and chemotherapy (Temozolomide – TMZ). TMZ is a DNA alkylating agent of the imidazotetrazine class [22], which acts by methylating guanine in guanine rich regions of DNA at N7 and, most importantly, at O6 positions, but also methylates N3 adenine. The cytotoxicity of TMZ is mediated through O6-methylguanine (O6-MeG) that, if left unrepaired, mispairs with thymine (instead of cytosine) during DNA replication, which results in persistent DNA strands breaks, prevention of replication and further G2/M cell cycle arrest in the second cycle after exposure to TMZ, ultimately leading to apoptosis. Of note, because TMZ is stable at acidic pH and gets labile at pH values above 7, it can be administered orally and become active preferentially at the brain tumor site, which is usually characterized by slightly more alkaline pH compared to the surrounding tissue [22].

The current standard treatment for patients diagnosed with GBM was employed after the results of a randomized, multicenter, phase III trial conducted by the European Organization for Research and Treatment of Cancer (EORTC) Brain Tumor and Radiotherapy Groups and the National Cancer Institute of Canada (NCIC) Clinical Trials Group in 2005 [23]. In this study the concomitant administration of TMZ (75 mg/m^2 per day) with fractionated radiotherapy (2 Gy/day, 5 days/week for 6 weeks, total dose of 60 Gy) followed by up to 6 cycles of adjuvant temozolomide (150-200 mg/m^2 per day, 5 days/week every 28 days) was compared with radiotherapy alone in patients with newly diagnosed GBM. The median survival of patients receiving RT with TMZ was 14.6 months compared to 12.1 months for patients treated with RT alone. The 2-year survival rate was 26.5% and 10.4% for RT plus TMZ and RT alone, respectively.

1.1.3 Molecular subtypes

Using the data from the Cancer Genome Atlas (TCGA) providing information on gene mutations, DNA copy number alterations and expression in a large GBM cohort, Verhaak et al. [24] performed unsupervised hierarchical clustering and identified four distinct molecular GBM subtypes: Proneural, Neural, Classical, and Mesenchymal (Figure 1.1). Classical subtype is characterized by the epidermal growth factor receptor (*EGFR*) amplification, *CDKN2A* and *PTEN* loss and lack of *TP53* mutations, while deletions and mutations of *NF1* gene predominantly occur in the Mesenchymal subtype. Alterations of platelet-derived growth factor receptor-α (*PDGFRA*), point mutations in isocitrate dehydrogenase 1 (*IDH1*), frequent *TP53* mutations and loss of heterozygosity are main features of the Proneural class. Finally, the Neural subtype expression patterns are the most similar to normal brain tissue.

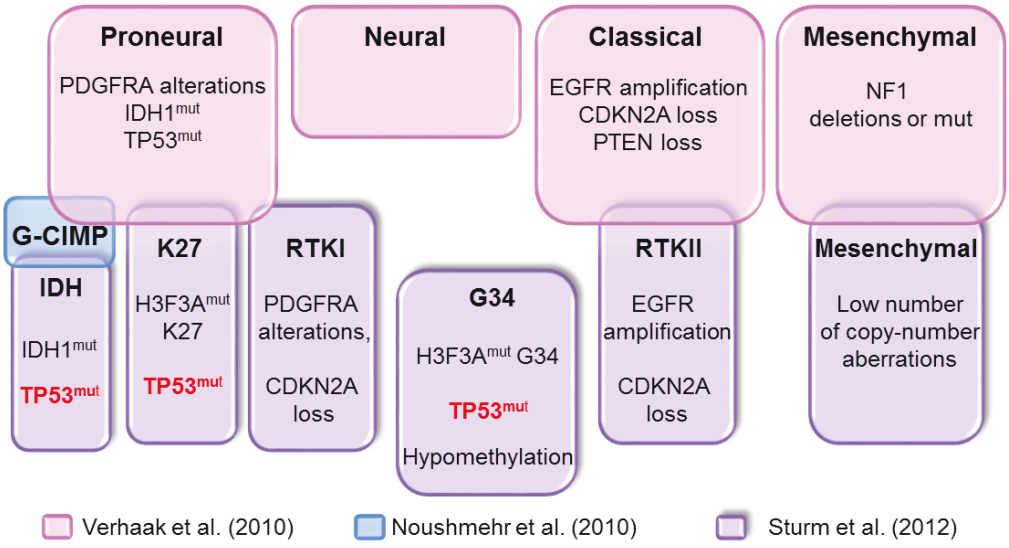

Figure 1.1. Expression and epigenetic GBM subgroups [24-26]

Further Noushmehr et al. [25] conducted profiling of TCGA GBM tumors according to their promoter DNA methylation alterations and found a distinct cluster of tumors possessing a CpG island methylator phenotype (G-CIMP). When compared to gene expression subtypes identified by Verhaak et al. [24], G-CIMP sample cluster was shown to be highly enriched for proneural GBM tumors and associated with IDH1 somatic mutations (Figure 1.1). In addition, patients with G-CIMP tumors were diagnosed at a significantly younger age (median age of 36) in comparison with non-G-CIMP tumors (median age of 59) and had better survival rate.

Further extending the analysis, Sturm et al. [26] investigated a cohort of GBM tumors from both children and adults and correlated their genome-wide DNA methylation patterns with mutations, DNA copy-number alteration and gene expression characteristics. This allowed identifying six epigenetic GBM subgroups: IDH, K27, G34, RTK I (PDGFRA), Mesenchymal, and RTK II (Classic). The IDH group was enriched for tumors with *IDH1* mutations and G-CIMP phenotype, confirming findings of Noushmehr et al. [25] and demonstrated Proneural gene expression pattern (Figure 1.1). The K27 and G34 subgroups included tumors with specific mutations in *H3F3A* gene encoding the replication-independent histone 3 variant H3.3, which result in K27 or G34 amino acid substitutions, respectively, thus affecting post-translational modifications of this protein. The tumors in G34 cluster were also characterized by genome-wide DNA hypomethylation. It is important to mention that *IDH1* and *H3F3A* mutations were shown to be mutually exclusive. In addition, the IDH, K27 and G34 subgroups were highly enriched in *TP53* mutations. *PDGFRA* amplification and Proneural expression displayed characteristics of RTK I (PDGFRA) cluster, in accordance with Verhaak classification [24]. Finally, RTK II (Classic) and Mesenchymal clusters corresponded to Classical and Mesenchymal gene expression profiles, respectively.

Coming back to the WHO classification into IDH-wild-type (primary) and IDH-mutant (secondary) GBM, *IDH1* mutations associated with the hypermethylation phenotype are the main molecular features of secondary GBM that distinguishes them from IDH-wild-type subtype (Figure 1.2). *TP53* mutations are detected in ~27% of IDH-wild-type and ~81% of IDH-mutant GBM [2]. The majority of GBM tumors with mutant *IDH1* are characterized by Proneural molecular signature, while wild-type *IDH1* GBM were found to be more heterogeneous and enriched in different expression profiles. GBM patients with mutant *IDH1* have a significantly longer median OS compared to patients with wild-type *IDH1* tumors, irrespective of the treatment regimen used: 24 months *versus* 9.9 months with RT or 31 months *versus* 15 months with RT+chemotherapy [2, 8]. Based on the evidence of differences in the types of gene alterations, the localization of the tumors in the brain, the clinical outcome of patients, etc. it was even suggested that primary and secondary GBM might develop from different precursor cells and, therefore, should be considered as distinct tumor types.

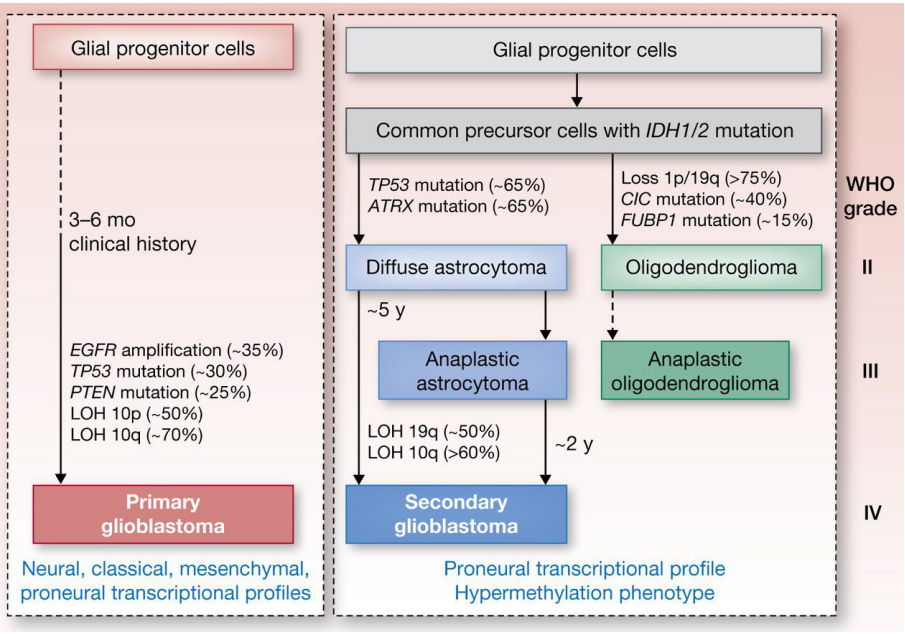

Figure 1.2. Development of primary and secondary GBM (adapted from [8])

The study by Brennan et al. [27] focused on molecular alterations affecting the core signaling networks in GBM. Using the TCGA data set the authors described the "landscape of somatic genomic alterations" in major cancer pathways: the p53 pathway (MDM family, *TP53*), PI3K-related pathway (*PIK3CA, PIK3R1, PTEN, EGFR, PDGFRA, NF1*) and the Rb pathway (*CDK4* and *6, CDKN2A/B, RB1*). This group found mutual exclusivity among alterations in certain genes within one pathway and between different pathways, for example mutations in *TP53* were mutually exclusive with amplifications of MDM family members or *CDKN2A*, while *PI3K* mutations were mutually exclusive with *PTEN* mutations or deletions, etc. When assigned to previously described GBM subgroups, *PDGFRA* amplification was confirmed to be enriched in hypomethylated Proneural subgroup, while *NF1* mutation was associated with Mesenchymal subtype.

More recently, Brown et al. analyzed TCGA Agilent microarray dataset to determine the relationship between expression of several extracellular stem cell markers and known molecular subtypes of GBM [28]. The rationale for this study was based on the widely accepted concept that GBM tumor recurrence is driven by a subpopulation of GBM cells with stem cell properties. CD133 is one of the most commonly used stem cell markers, although some reports suggested that non-stem glioma cells also can express CD133. In their study, Brown and colleagues found that the CD133 module (top 5% of genes significantly positively correlated with this stem cell marker) was enriched with Proneural molecular subtype and negatively correlated with the CD44 (another stem cell marker) module, which, in turn, was enriched in the Mesenchymal subtype of GBM. Therefore, the authors concluded that the sets of genes that co-express with specific cell stem markers in tumor cells can be used for determination of the GBM molecular subtype independently of signatures of cancer stem cells.

1.1.4 GBM heterogeneity and the role of cancer stem cells

The classification of GBM is hampered by its extreme intratumoral heterogeneity – the presence of tumor cell populations with different mutations, chromosome aberrations and gene expression signatures within the same tumor. GBM heterogeneity can be regional, in which specific alterations are detected in certain regions of the tumor (e.g. amplification of *EGFR* at the invading edge of the tumor), and/ or mosaic, when cell subpopulations with different alterations create a mosaic without any evident organization [29]. GBM heterogeneity is believed to be largely responsible for tumor resistance to chemo- and radiotherapy. There are two major models of how the heterogeneous tumors composed of tumor cells with different molecular characteristics are formed: clonal evolution (stochastic) and cancer stem cell (CSC) models (Figure 1.3).

According to *clonal evolution model* the spontaneous somatic mutation(s) occur in an individual cell, which makes it neoplastic, and gives the growth advantage over surrounding cells [30]. In the population of cells formed as a result of proliferation of the original neoplastic cell some of the cells would acquire additional mutations, that are beneficial in particular microenvironment and therefore would be selected for [31]. Over time, the sequential selection of subclones will give rise to heterogeneous cell populations within the same tumor. The assumption that all tumor cells possessing beneficial mutations have a similar potential for regenerating tumor growth is the major distinctive feature of this model [32]. The components of microenvironment, according to the clonal evolution model of intra-tumor heterogeneity, are main effectors for formation of different subclones. These include regulations via a) the autocrine, paracrine secretions and cell-cell interactions with surrounding cells (stromal, endothelial, etc.), b) collaboration between different subclones within the same tumor; c) physical factors – pressure,

anatomy and accessibility of blood vessels supplying oxygen and nutrients, which mostly result in regional heterogeneity; d) chemotherapeutic agents and radiotherapy used for treatment.

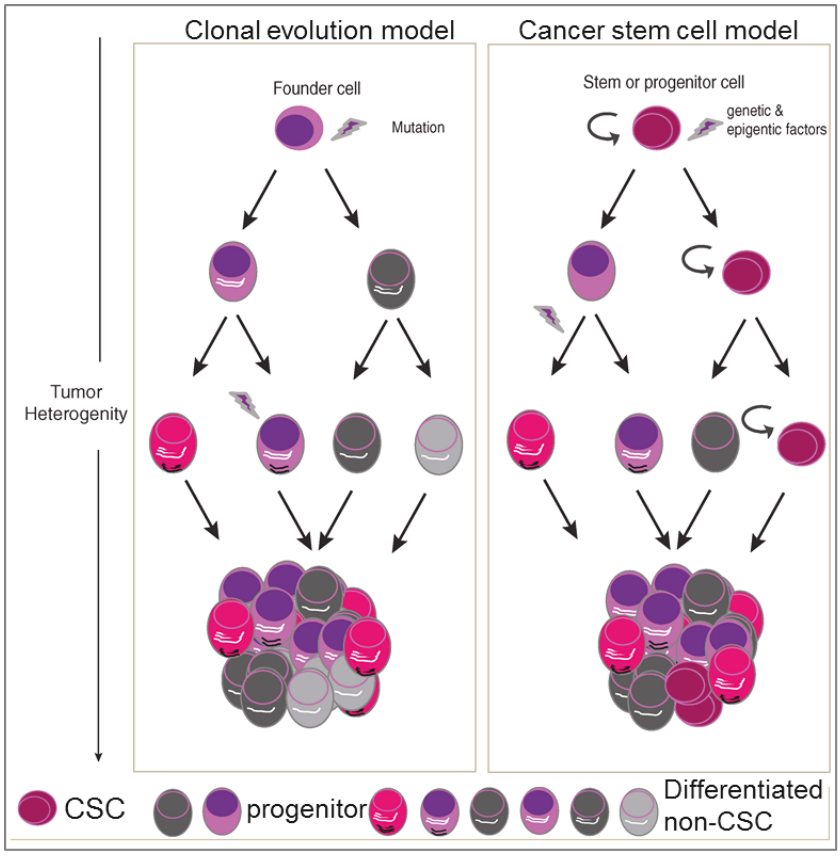

Figure 1.3. Models of tumorigenesis: clonal evolution and cancer stem cell (CSC) models (adapted from [33])

Prior to describing the cancer stem cell model, we need to provide the definition of cancer stem cells (CSCs) in general and, more specifically, of glioma stem cells (GSCs). CSCs are considered to be a driving force for tumorigenesis and are defined as a small population of cells in the tumor that resemble the properties of normal stem cells ("stem-like" properties), such as the ability to perpetuate themselves through self-renewal and to generate diverse differentiated cells [34]. However, CSCs cannot be considered multipotent, as normal SCs, because they are

transformed and produce genetically abnormal progeny [35]. Due to the histological heterogeneity of GBM tumors as well as their highly invasive and aggressive behavior it was suggested that GBM may originate from the CSCs. The CSC, in turn, may derive from either the transformed pluripotent neural stem cell or from the restricted progenitors, or even differentiated cells that acquired ability for self-renewal. The existence of brain tumor stem cells (BTSCs) and, specifically, GSCs was first demonstrated more than a decade ago [36, 37], when the investigators were able to isolate tumor cells with properties similar to neural stem cells (NSC) from the human brain tumors and showed their ability to form neurospheres, when grown under serum-free conditions *in vitro* in the presence of mitogens (EGF, FGF2). When grown *in vitro*, GSCs were also shown to express several NSC markers, such as CD133, SSEA1, NESTIN, SOX2, BMI1 and MUSASHI [38]. GSCs are defined as cell population in glioma tumor that is characterized by: a) self-renewal capacity, b) ability to form tumor upon orthotopic transplant *in vivo*, and c) ability to generate differentiated neuron-like and glia-like progeny [35, 39]. Slow cell cycle progression is another attribute of GSC populations within the tumor that distinguishes them from populations of rapidly proliferating differentiated cells forming the tumor bulk, and predispose them to radio- and chemoresistance, which will be described in more details below.

The *CSC model*, in contrast to clonal evolution model, describes the heterogeneity as a consequence of the asymmetric division of CSCs generating two types of daughter cells: stem cells and differentiated cells [29]. The differentiated cells would form the bulk of the tumor and have a limited proliferative potential, while a small population of CSCs would remain tumorigenic. This hypothesis is confirmed by the reports that only a small population of tumor cells are able to initiate tumor formation upon transplantation into immunodeficient mice. Moreover, these tumorigenic cells can be distinguished from other cancer cells forming the bulk of the tumor based on their

specific surface markers [40]. It is necessary to note that two models of tumor heterogeneity are not mutually exclusive as the microenvironment can affect CSCs, thus, selecting them according to the clonal evolution model.

The factor of cooperation between different clones within the tumor has been suggested in the branched evolution and interclonal cooperativity models [29, 41, 42]. According to the branched evolution theory all daughter clones carry the mutations of the original clone, which are called "driver" mutations and are crucial for malignancy and tumor growth. At the same time, daughter clones acquire new mutations, which are referred to as "passenger" mutations and are advantageous only in particular tumor microenvironment, but can also become "driver" mutations under certain conditions. As a result, multiple subclones can compete with each other and/ or evolve in parallel. Furthermore, the interclonal cooperativity model suggests that different clones can "cooperate" with each other by, for example, secreting the extracellular factors that change the microenvironment in a way that it is most beneficial for tumor maintenance and progression.

The heterogeneity of GBM tumor cells is in majority represented by genetic alterations in receptor tyrosine kinases (RTK), which were detected in 67% of primary (*de novo*) GBM tumors in TCGA [27]. In particular, 57% of GBM samples harbored alteration in *EGFR*, 13% - in *PDGFRA* and 2% - in *MET*, while 42% of tumors with *PDGFRA* alteration and majority of tumors with *MET* alteration were characterized with the concurrent alterations in *EGFR*. One of the recently developed methods to study the intratumoral (subclonal) heterogeneity of GBM is the single cell-derived clonal analysis. In accordance with previous reports from bulk GBM tumors, this technique showed that *EGFR* amplification was a frequent event in subclones [43], while the expression of *PTEN* and *EGFR* was heterogeneous among different single cell-derived clones from the same patient tumor. Furthermore, these clones responded differently to TMZ and other known

anti-cancer drugs. However, clones from the same tumor were found to be more genetically similar to each other than to those from other tumors. Clearly, additional studies are needed to obtain a better understanding of the mechanisms of GBM heterogeneity and overcome its consequences, such as resistance to radiotherapy and chemotherapeutic agents.

1.1.5 Mechanisms of GBM radio- and chemoresistance

Radioresistance. Although being a key component of standard treatment for patients diagnosed with GBM, RT alone does not improve their survival rate, suggesting that GBM possesses effective adaptive mechanisms to resist effects of IR. The described above CSCs and heterogeneity of GBM tumors play an important role in treatment failure. GBM cells in general and, to a greater extent, GSCs were shown to be intrinsically radioresistant, while the use of RT can lead to appearance of new mutations and epigenetic modifications, thus, killing radiosensitive cells and selecting for cells with acquired radioresistant properties [44]. Indeed, it was reported by Tamura et al. [45] that CD133+ GSCs were enriched in GBM specimens obtained after high-dose irradiation with Gamma Knife surgery and external beam radiation therapy, but not prior to treatment, which indicates that GSCs are characterized by high radioresistance and are, therefore, responsible for tumor recurrence. The intrinsic mechanisms of GBM radioresistance include activation of specific checkpoint proteins required for cell cycle arrest, increased efficiency of DNA repair machinery and activation of signaling pathways promoting cell survival. Extrinsic mechanisms of resistance to RT, in turn, include maintenance of hypoxic niche for CSCs and, on the other hand, promotion of tumor angiogenesis in order to meet oxygen demand for tumor growth.

DNA double strand breaks (DSBs) can be induced by IR, either via its direct action or indirectly as a result of ionization of water molecules and generation of free radicals. Formation of DSBs triggers the DNA-damage response (DDR) within the cell and activation of DNA repair pathways, such as homologous recombination repair (HRR), nonhomologous end-joining (NHEJ) or an alternative NHEJ pathway. In a simplified model of NHEJ, the DSBs are detected by Ku70/80 heterodimer, then the non-ligatable DNA termini are tethered by the Ku/DNA-PKcs (DNA-dependent protein kinase catalytic subunit) complex, processed by specific enzymes and the DSB ends are ligated [46]. NHEJ is a complex and error-prone process, which often leads to mutations and chromosomal rearrangements [47]. Alternative NHEJ occurs in the absence of classical NHEJ, and thus is suggested to have backup functions. The HRR DNA repair, in turn, utilizes homologous DNA sequence as a template and therefore is more accurate and can occur only after DNA replication [48]. The choice of repair pathway depends on a number of factors [49]: a) the stage of cell cycle – as the HRR is active only in S and G2 phases, while NHEJ works throughout the cell cycle, b) the type of resection of DNA ends, and c) the phosphorylation status of DNA-PK – a major protein in NHEJ [50].

The effectiveness of DNA repair determines the ability of tumor cell to survive and resist apoptosis, therefore, the findings of increased capability of GSCs for efficient HRR indicate that this may be one of the mechanisms of GBM radioresistance [51]. In addition, rapid activation of checkpoint proteins leading to cell cycle arrest can potentially allow for more efficient DNA-damage repair and increased survival. It was shown [52] that GBM CSCs can preferentially activate checkpoint responses at early time points following exposure to IR through phosphorylation of ataxia-telangiectasia-mutated (ATM), Rad17, Chk1 and Chk2 checkpoint proteins to a greater extent compared to non-stem tumor cells. The quiescent state of CSCs

associated with slow cell proliferation and growth also contributes to their radioresistance, as IR is known to mostly target rapidly proliferating cells with the greatest effect in G2/M phase and the lowest – in S phase [53]. In contrast, another study [54] that compared response of GBM CSCs and established glioma cell lines to IR, demonstrated that CSCs were more sensitive to radiation than cell lines and their double-stranded breaks repair capacity was reduced. In addition, Squatrito et al. [55] showed that Chk2 is required for glioma response to IR, while its loss abrogates the activation of checkpoints induced by DNA damage and protects GBM cells from apoptosis, therefore potency of its inhibition for overcoming glioma radioresistance suggested by Bao et al. [52] is debated. The inconsistency between results of these and other studies could possibly be due to the lack of universal surface markers allowing to isolate and study radioresistance of the pure populations of CSCs. The most commonly used marker, transmembrane glycoprotein CD133 (Prominin-1), is also found on the surface of hematopoietic SCs, endothelial precursors and NSCs. The results of a number of studies put the use of CD133 as a reliable marker for GSC isolation in question [38]. In one study, CD133- GBM cells were shown to have tumorigenic potential in nude rats, while several other studies reported that CD133+ cells are not always present in the freshly obtained GBM specimens and suggested an alternative marker - SSEA1/CD15. In addition, extreme GBM heterogeneity, discussed above, makes studying of radioresistance even more challenging, as GBM cells characterized by distinct molecular profiles may differ in their DNA damage repair proficiency [56] and, therefore, show various levels of radiosensitivity.

Another factor of intrinsic radioresistance in GBM includes activation of signaling pathways, which prevent apoptosis and promote cell survival in response to IR. In particular, activation of PI3K/Akt signaling through phosphorylation of PI3K and Akt in tumor cells from glioma patients was shown to have inverse relationship with levels of cleaved caspase 3, thus, demonstrating the

role of this pathway in inhibition of apoptosis [57]. In addition, activation of these proteins also had a prognostic significance, correlating with adverse outcome in glioma patients and in a subset of patients with GBM treated with RT alone. Further, Chautard et al. reported that suppressing Akt with chemical inhibitor enhanced radiosensitivity of glioma cell lines in a clonogenic survival assay [58].

Additionally, c-Jun N-terminal kinase (JNK) signaling was shown by Yoon et al. [59] to be important for stem-like properties of sphere-cultured glioma cells (also described in stem-like GBM cells by [60]) and for increased expression of Notch-2 in both glioma cells and patient derived primary glioma stem-like cells. Activation of JNK, dependent on PI3K phosphorylation, was demonstrated to be involved in response to IR and its inhibition led to increased cell death in glioma spheres. Importantly, Notch-1 and Notch-2 activation was also reported to protect GSCs from IR-induced cell death [61]. Other signaling molecules and pathways involved in response to IR have been investigated and suggested as candidates for targeted therapy include the transcription factor nuclear factor-kappa B (NF-κB) [62-64] and TGF-β [65-67].

Aside from intrinsic factors described above, GBM response to RT also depends on the tumor microenvironment, in particular the proximity of vasculature. In the case of rapidly proliferating cancer cells, the tumor mass is increasing at a rate which cannot be accompanied by formation of appropriate amount of new blood vessels sufficient to supply all tumor cells with oxygen. Therefore, due to a limited distance at which the oxygen can diffuse, a central region of GBM tumors is often characterized by the presence of necrotic cells and hypoxic conditions. The so-called hypoxic niche with low concentrations of oxygen (0.1-2.5%) [68, 69] leads to an increase in expression of hypoxia markers, such as hypoxia inducible factor (HIF) family proteins. Under hypoxia conditions they form the HIF transcriptional complex (HIF-1α/β), which can induce

expression of multiple target genes, most importantly molecules and factors promoting angiogenesis, cell growth, survival and invasiveness. Levels of expression of HIFs were also reported to affect glioma patients outcome [70]. In addition, it was shown that low oxygen levels help to maintain cancer cell stemness and prevent cell differentiation [71-73]. Moreover, tumor control following RT was shown to negatively correlate with tumor hypoxia [74]. It thus can be suggested that hypoxia within the tumor mass might prevent formation of oxygen free radicals following exposure to IR, protect cells from the ROS-induced DNA damage and assist in maintaining the population of CSCs, while hypoxia-induced activation of HIF-1α/β increases levels of expression of specific genes promoting angiogenesis, metabolic reprogramming of cells and increase in tumor mass. Another, less studied, effect of microenvironment on the CSCs response to IR may occur via cellular interactions, either directly or through the release of various soluble factors promoting radio- and chemoresistance [75].

Chemoresistance. The cytotoxicity of TMZ that occurs through methylation of O6 guanine residues (O6-MeG) can be inhibited or abrogated by two well-described mechanisms. First and foremost expression of O6-methylguanine DNA methyltransferase (MGMT) DNA repair protein, which acts by removing the alkyl groups from the O6 position of guanine and, thus, interferes with cytotoxicity of TMZ [22, 76]. The role of MGMT in tumor response to treatment and prognosis for GBM patients will be described in more details below.

The second mechanism of resistance to TMZ is mediated through the deficiency in the DNA mismatch repair (MMR) pathway. MMR functions by recognizing the insertion/deletion loops or base-base mismatches in newly synthesized DNA strands [77] and degrading the section of the strand containing the error, therefore, allowing its replacement with the correct sequence by DNA polymerase based on the template DNA strand. In case of mutations in the MMR pathway leading

to its inactivity, the O6-MeG–thymine mispairs are not recognized and repaired, which can result in completion of DNA replication, cell cycle progression and avoidance of apoptosis.

In addition to MGMT activity and defects in MMR, TMZ cytotoxicity can also be affected by base excision repair (BER) – a pathway important for repair of damaged bases and DNA single-strand breaks (SSBs) [22, 76]. In particular, BER can repair guanine and adenine methylated by TMZ (as described earlier) at N7 and N3 positions, respectively. Although these adducts contribute less to cytotoxicity of TMZ compared to O6-MeG, they can become more important when the O6-MeG are efficiently repaired by MGMT or are not recognized due to MMR deficiency. Therefore, BER as well as MGMT activity can serve as therapeutic targets in order to enhance TMZ cytotoxicity and increase its efficacy.

1.2 MGMT AND p53 IN GBM

1.2.1 MGMT and its role in GBM

MGMT, also sometimes referred to as O6-alkylguanine DNA alkyltransferase (AGT), is a small protein, which repairs the mutagenic DNA lesion, O6-methylguanine. Specifically, MGMT acts by removing the O6-alkylguanine adduct in DNA and transferring it to the cysteine residue (Cys 145) within its active site, thus, producing S-methylcysteine (Figure 1.4) [78, 79]. Formation of S-methylcysteine is accompanied by loss of MGMT activity and MGMT is therefore described to act via the suicide mechanism, as its inactivation following the formation of S-methylcysteine leads to degradation of the protein via the ubiquitin proteolytic pathway [80, 81]. As previously mentioned, DNA methylation at O6 position of guanine (O6-MeG) can occur as a result of exposure to chemical agents, and O6-MeG repair in normal cells is a protective mechanism against its mutagenic and carcinogenic properties.

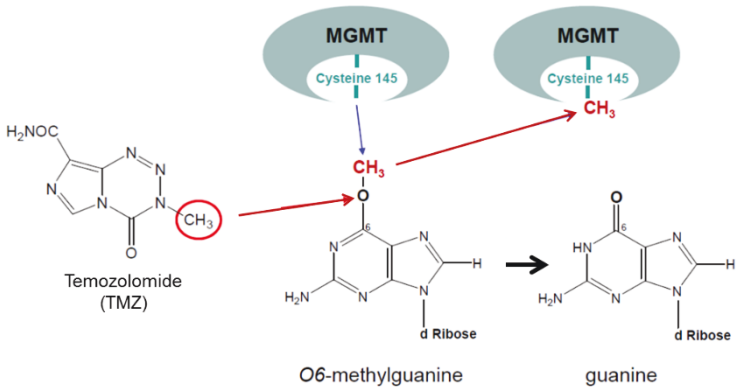

Figure 1.4. Mechanism of action of MGMT protein (adapted from [22]).

Because one MGMT molecule is necessary to repair one methyl adduct, the capacity of the cell to repair O6-MeG largely depends on levels of MGMT expression, which can be highly variable in different cell types, tissues, phases of cell cycle and vary among individuals and with age. In addition to humans, alkyltransferase activity was reported in the cells of various species, e.g. in *E.coli*, rodents (mouse, hamsters, rats), monkeys, etc. [82, 83]. When measured in normal adult human tissue extracts, the highest MGMT activity was detected in liver and showed 8-fold inter-individual variation [84]. MGMT activity in stomach was similar to that in small intestine and colon tissues, and the inter-individual variation in two latter tissues was even larger than in the liver. Furthermore, two examined colon tumors were characterized by several fold higher activity than normal colon mucosa obtained from the same patients. MGMT activity was also detected in normal human brain tissues, although found to be much lower compared to that in the tissue from other organs, especially liver [85]. Human brain tumor tissues, in turn, had a trend towards higher MGMT activity in comparison with normal brain tissue, but showed much variation between samples. MGMT activity, thus, is an important mechanism of DNA repair in normal cells, while, as previously stated, such activity in cancer cells interferes with cytotoxicity of alkylating agents, such as TMZ that is used as chemotherapy in patients with GBM.

MGMT gene, located on chromosome 10 (10q26.3) is transcribed into a transcript consisting of 5 exons and encoding 238 amino acid protein. An important characteristic of MGMT gene is the presence of 97 CpG sites within the CpG island (containing CG sequences with high frequency) in the proximal promoter region and the first exon. The methylation of CpG sites in promoter region of MGMT gene is the main mechanism of MGMT expression regulation. Importantly, *MGMT* gene silencing by its promoter methylation was shown to be associated with better prognosis for GBM patients (median overall survival of 18.2 months) [86] compared to patients possessing tumors with unmethylated *MGMT* promoter (12.2 months), irrespective of treatment regimen (TMZ plus RT or RT alone). Furthermore, GBM patients with promoter methylation benefited from the combination of RT and TMZ (21.7 months) in comparison with RT alone (15.3 months), while for patients with unmethylated tumors there was no striking difference between median overall survival in groups receiving combination (12.7 months) or RT alone (11.8 months).

Despite being a relevant predictive biomarker in GBM, *MGMT* gene promoter methylation status is sometimes difficult to interpret. First, the extent of methylation throughout 97 CpG sites can be highly variable, and methylation of certain CpG sites might affect MGMT expression to a greater extent than that of others [87, 88]. In support of this observation, it was reported that methylation status determined by methylation-specific PCR does not always correspond to the levels of MGMT protein detected by IHC staining / Western blotting [89-91] or mRNA levels [92]. Clearly, there are other mechanisms of regulation of MGMT expression in addition to promoter methylation (listed further). Therefore, MGMT mRNA quantitation instead of or in addition to *MGMT* promoter methylation status analysis has been proposed as a useful prognostic biomarker in GBM [93]. The second difficulty pertains to the fact that MGMT methylation status in GBM cells within the same tumor is frequently different, for example depending on the

accessibility of oxygen supply or other unknown factors, which further contributes to the issue of GBM heterogeneity. Third, there are different opinions on whether *MGMT* promoter methylation is really a predictive (response to TMZ) rather than prognostic (survival) biomarker. Importantly, in a large randomized phase III clinical trial for patients with anaplastic glioma [94], hypermethylation of the MGMT promoter was shown to be associated with prolonged progression-free survival (PFS) in patients receiving radiotherapy alone (without alkylating agents). In another study [95], methylation of certain CpG regions was shown to be strongly associated with better patient survival in glioma CpG island methylator phenotype (G-CIMP+) group. Finally, it is not clear whether methylation of *MGMT* promoter or the levels of MGMT expression (mRNA, protein) is superior as a predictive marker in GBM patients.

Other identified mechanisms regulating MGMT expression include the activity of various transcription factors (TFs), the microRNAs and histone modifications. A number of TFs were shown to be able to bind specific sequences in the promoter region of MGMT, such as Sp1 [96, 97], NF-κB [98, 99], CBP/p300 [100], AP-1 and AP-2 [101]. MGMT was also shown to possess glucocorticoid responsive elements (GRE) within its promoter, therefore it can be induced by glucocorticoids [102, 103]. Grombacher et al. [103] also demonstrated that IR induced the transcriptional activation of MGMT in rat hepatoma cells and suggested it might be a DNA-damage inducible gene, however such activation might be cell-type and context specific.

As previously mentioned, MGMT expression often varies throughout different regions of the GBM tumor and is frequently found to be increased in the hypoxic central core [104], particularly in the population of stem cells [105] resistant to TMZ. Furthermore, the existence of MGMT/HIF-1α axis under hypoxic conditions was suggested. This hypothesis was supported by the findings that inhibition of HIF-1α expression induced by the bone morphogenetic protein

BMP-2 [71], was accompanied by downregulation of MGMT in GBM stem-like cells and led to their sensitization to TMZ [106]. In conclusion, despite the identification of multiple TFs involved in regulation of MGMT transcription, exact understanding of the coordination of their activity for activation of MGMT expression is still lacking.

MicroRNAs (miRs), small non-coding RNAs that target specific binding sites in the 3′ untranslated region (UTR) of protein-encoding mRNAs [107], are another type of factors that can regulate MGMT expression. Zhang et al. [108] demonstrated that miR-181d inversely correlated with overall survival in 82 GBM patients as well as in TCGA GBM dataset (n= 424) and in an independent cohort of 35 patients with GBM. Moreover, this study showed that MGMT 3′UTR is a direct target of miR-181d *in vitro* and that miR-181d expression inversely correlates with MGMT transcript level in TCGA dataset, even in specimens with unmethylated *MGMT* promoter. Another study [109] confirmed the effect of miR-181d and also miR-767-3p on MGMT mRNA and protein levels *in vitro* and further determined their relevance as predictors of MGMT mRNA expression in TCGA GBM dataset. Interestingly, miR-648 downregulated MGMT protein expression *in vitro*, but did not influence its mRNA levels and, thus, had no predictive value in TCGA. In summary, it appears that miR-181d and miR-767-3p lead to MGMT mRNA degradation, while miR-648 inhibits translation of MGMT protein. In a recent study [110] Kushwaha et al., showed that miR-603 bound MGMT 3′UTR and suppressed MGMT mRNA and protein expression *in vitro* and *in vivo*. In addition, miR-603 as well as miR-181d inversely correlated with MGMT expression in clinical glioblastoma specimens (n=74). Quintavalle et al. [111] reported that miR-221 and miR-222 paralogues, frequently upregulated in GBM patients, targeted MGMT mRNA and downregulated both mRNA and protein levels *in vitro*. Also a small group of GBM patients (n=11) with unmethylated MGMT and a survival of less than 15 months (short survival) was characterized

by higher levels of miR-221 and lower levels of MGMT compared to the group with long survival (>15 months). In summary, thus far, at least 6 microRNAs have been reported to affect MGMT expression in GBM, and with more and more continuously emerging new data, it is clear that the role of microRNAs in MGMT regulation is far from being fully discovered.

Histone modifications are another type of epigenetic regulation of MGMT expression, in addition to promoter methylation. In particular, increased acetylation of lysine on histones H3 and H4 (H3Ac and H4Ac) was associated with relaxed chromatin and transcriptional activity as well as high levels of MGMT *in vitro* [112]. In contrast, di-methylation of lysine on histone 3 (H3me2K9) led to inhibition of MGMT expression [112, 113]. These findings were supported by the study showing that histone deacetylases (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) induced expression of MGMT and increased resistance to TMZ *in vivo* [114]. The effect of treatment based on HDAC inhibitors on GBM, which may influence expression of additional genes unrelated to MGMT regulation, is extensively investigated [115-117].

Because MGMT repairs lesions caused by alkylating agents (TMZ) and is, therefore, largely responsible for resistance of GBM tumors to TMZ, the approaches modulating MGMT expression or activity in order to improve GBM response to chemotherapy have been under scrutiny. O6-benzylguanine (O6-BG) was shown to inactivate and deplete MGMT levels by acting as its alternate substrate, leading to the transfer of benzyl group to the cysteine residue at MGMT active site [118]. O6-BG-induced depletion of MGMT activity was found to be more effective than that by O6-methylguanine. Although O6-BG-mediated decrease in MGMT activity is transient, it ought to be sufficient to enhance the cytotoxicity of alkylating agents. In order to assess safety and efficacy of O6-BG in sensitizing tumors to TMZ and also to determine the dosing schedule and maximum-tolerated dose, phase I and II clinical trials were conducted in patients with progressive

or recurrent TMZ-resistant anaplastic glioma or GBM tumors, respectively [119, 120]. Although showing efficacy in anaplastic glioma, the combination of O6-BG with TMZ did not improve the response of TMZ-resistant GBM (only 1 responder of 34 patients with GBM) and the progression-free (PFS) and overall (OS) survival of GBM patients, while the toxicity to the hematopoietic system (myelosuppression) was observed in nearly half of the patients enrolled.

In addition, several studies reported the ability of interferon-β (IFN-β) to inhibit MGMT expression in glioma cells, possibly through wild-type (wt) p53 induction, leading to their increased sensitivity to TMZ *in vitro* [121] and *in vivo* [122]. However, the addition of IFN-β to the standard RT/TMZ regimen for newly diagnosed GBM patients did not improve their PFS or OS, while causing an increased incidence of adverse effects, when assessed in phase II clinical trial in Japan [123], and, therefore, was not recommended for further investigation in this category of patients. A recent study reported a prolonged survival as result of using IFN-β and TMZ combination therapy in patients with recurrent malignant gliomas [124], although the small number of patients (7 cases) is obviously a major limitation of the study.

Another suggested strategy for inhibition of MGMT involves suppression of STAT3, a cytoplasmic transcription factor commonly activated in many types of tumors due to its pro-survival activity, which is essential for tumor growth. The abundance of STAT3 in its activated phosphorylated form (p-STAT3) was shown to correlate with brain tumor grade, whereas STAT3 was suggested to be involved in GBM progression [125]. Importantly, p-STAT3 correlated with MGMT expression levels in malignant glioma specimens, while STAT3 inhibition in GBM cell lines downregulated MGMT and increased their sensitivity to TMZ [126]. Furthermore, in their work Bobustuc et al. showed that the antiepileptic drug, levetiracetam, was able to inhibit both mRNA and protein expression of MGMT in glioma cell lines by recruiting the mSin3A/histone

deacetylase 1 (HDAC1) corepressor complex and promoting mutant (mut) p53 binding to the promoter of *MGMT* [127]. Decrease in MGMT expression was also observed in newly diagnosed GBM patients treated with this drug. It is, therefore, possible that the antiepileptic drugs, commonly used for treatment of seizures in glioma, could sensitize tumor cells to temozolomide, thus, providing additional benefit for patients.

1.2.2 p53: structure, functions and mutations

The p53 protein was first described in 1979 as a transformation-related antigen in mouse cancer models [128]. It was later found that in this study p53 protein was in its mutant state, while other studies demonstrated that wtp53 actually acted as a tumor suppressor, preventing the transformation of cells [129], and was even named a "guardian of the genome" [130]. p53 is a transcription factor and a member of protein family that also includes p63 and p73 proteins, which are two evolutionary older homologues of p53 and share the same domain structure [131].

The structure of p53. The human p53 protein is encoded by a 20 Kb *TP53* gene, containing 11 exons and a 10 kb intron between the first and the second exons [132] and located on the short arm of chromosome 17 (17p13) [133, 134]. In its wild-type state p53 protein (393 amino acids) consists of the following domains: N-terminus, a central core and a C-terminal region (Figure 1.5) [135-137]. The N-terminus (residues 1-92) contains an amino-terminal domain, which comprises two transcriptional activation domains (TADs), TAD1 and TAD2, and is involved in the interaction with other proteins, such as MDM2, CBP/p300, etc., and a proline-rich region that is considered to play a regulatory role by preventing MDM2-induced degradation of p53 and, thus, maintaining its stability. The central core domain (residues 94-292) is responsible for binding to target DNA containing the specific consensus sequence (DNA-binding domain). Finally, the C-

terminus (residues 301-393) contains an oligomerization (tetramerization) domain, the negative regulatory domain that affects DNA-binding activity of p53, as well as nuclear localization signal and leucine-rich nuclear export signal sequences.

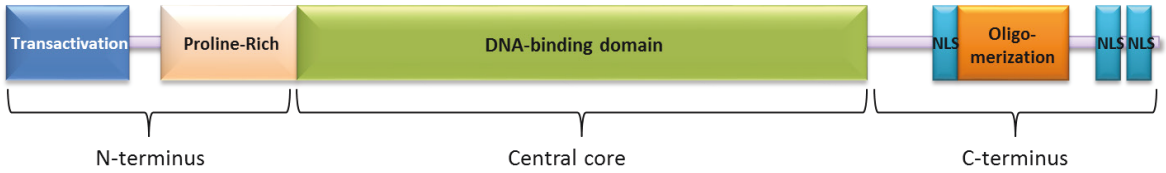

Figure 1.5. The domain organization of human p53 protein [135-137]

Wild-type p53 functions. p53 tumor suppressor is activated in response to genotoxic stress (e.g. DNA damage following exposure to IR, chemotherapy as well as infections, heat or cold shock, mitotic spindle damage, hypoxia, unfolded proteins, improper ribosomal biogenesis, telomere shortening or oncogene overexpression) and acts as a transcription factor regulating expression of hundreds of target genes (Figure 1.6). p53 protein binds to sequence-specific p53 response elements of target genes as a tetramer, which is formed under the control of tetramerization domain in the C-terminus of the protein [137, 138]. Upon binding to DNA, p53 can induce or suppress transcription of many genes, such as *Bax*, *Fas*, *Noxa*, *PUMA*, *CDKN1A*/*p21*, etc., the choice of which is dependent on the cell type, the nature of stress and microenvironment. The p53-induced activation or repression of certain genes or microRNAs (miR-34, miR-192, miR-194, miR-215, etc.) [139] determines the cell fate, leading to different responses such as cell cycle arrest, DNA repair, apoptosis, or senescence. The importance of p53 was also reported for cell survival and regulation of oxidative stress, invasion, motility, autophagy, angiogenesis [140], differentiation, etc. [141].

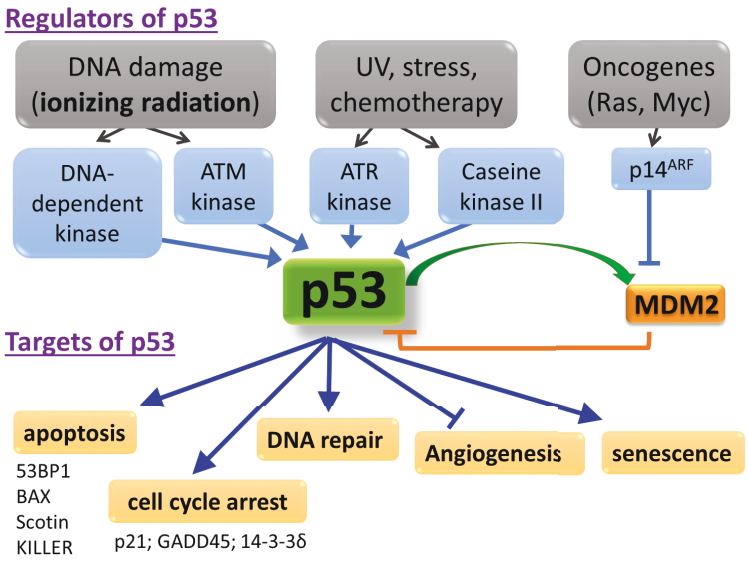

Figure 1.6. Regulators and downstream targets of p53 [137, 138, 140, 141]

Regulation of p53. MDM2 is one of the main regulators of wtp53 levels in the cell. By binding to TAD of p53 this E3 ubiquitin ligase sterically inhibits protein transactivation and also suppresses p53 stability by promoting its ubiquitination and subsequent proteasomal degradation. This occurs under conditions when wtp53 activity is not required and is unfavorable for cell viability. In contrast, upon stress stimuli the interaction between MDM2 and p53 is disrupted via multiple post-translational modifications of p53 and MDM2 and p53 becomes activated. *MDM2* itself is a p53 target gene and increase in its expression allows maintaining low levels of p53 under normal physiological conditions. MDMX (also called MDM4), MDM2-family member, is another negative regulator of p53. Upon dimerization with MDM2 it facilitates E3 ligase activity of the latter, thus, contributing to p53 degradation [142]. Other negative regulators of p53 include Pirh2, COP1, CHIP, ARF-BP1, E6-AP, TOPORS, TRIM24 and MKRN1 ubiquitin ligases, although their effect on p53 stability is not as prominent as that of MDM2 [143].

Two proteins, p300 and CBP, are also involved in regulation of p53 activity. However, the mode of this regulation is context specific – in some cases these proteins promote MDM2-mediated ubiquitination of p53 and its subsequent degradation, while in other cases they acetylate lysine residues of C-terminus of p53, thus, preventing its ubiquitination and helping to maintain stability of p53 protein. It was shown that the choice between two mutually exclusive roles of p300/CBP is to a great extent determined by post-translational modifications of p53 protein [143], which would be described in more detail below. In the p300/CBP context, phosphorylation of specific residues in the N-terminal region of p53 (Ser15, -20, -33, -37, -46, -55, and Thr18) induces p300/CBP binding and promotes p53 transactivation, while binding of MDM2 is blocked.

Recently, miRs were shown to be involved in p53 pathway, not only as p53 targets, but also as regulators of p53 or other components of the p53 pathway [144]. For example, miR-29 inhibited expression of CDC42 (a member of the Rho family of GTPases) and the regulatory subunit of phosphatidylinositol-3 kinase (PI3K), leading to p53 activation and apoptosis in human breast adenocarcinoma, colon and gastric carcinoma cell lines. In addition, p53 was identified as one of the targets of two isoforms of miRNA-125 - miR-125a and miR-125b, which were shown to downregulate p53 and suppress apoptosis [145].

Activation of p53. Depending on the nature of stress, several pathways can become activated and trigger p53 response in the cell by stabilizing p53 protein and preventing its degradation [146]. One of the pathways is activated by DNA damage and DNA double-strand breaks, in particular, such as caused by IR, leading to stimulation of ATM, Chk1 and Chk2 protein kinases. Phosphorylation of amino-terminal sites of p53 performed by these kinases prevents MDM2 binding to p53 and stabilizes the protein. The second pathway capable of activating the p53 network is dependent on $p14^{ARF}$ and responds to aberrant growth signals that can arise from Ras

or Myc oncogenes expression. $p14^{ARF}$ is able to inhibit MDM2 activity by binding it and causing its transfer to the nucleolus sub-compartment within the nucleus, thus, eliminating the possibility of its interaction with p53. Two kinases - ATR (ataxia telangiectasia related) and casein kinase II are main proteins regulating the third pathway, which is triggered by protein-kinase inhibitors, UV light or chemotherapeutic agents, and leads to stabilization of p53.

Post-translational modifications (PTMs) of p53. In response to various stress stimuli, some of which were described above, p53 is activated and undergoes post-translational modifications, such as phosphorylation, acetylation, sumoylation, methylation, ubiquitination, neddylation, poly-ADP-ribosylation, nitration, etc. [147]. Discovery and characterization of more than 50 individual PTMs of p53 allows formulation of their main features: *i)* interdependent nature of PTMs - cooperative activity of multiple modifications for promoting subsequent cascade of events, rather than specific functions attributed to each individual modification, for example phosphorylation of serine 15 is required for sequential modification of other residues; *ii)* depending on the nature of stress different residues can be modified and the type of their modification also varies, for example, activation of p53 through $p14^{ARF}$ pathway leads to its acetylation, rather than phosphorylation. It is important to note that such modifications as acetylation and ubiquitynation are mutually exclusive, and so are methylation and neddylation [143]. The role of PTMs includes, but is not limited to: *a)* promotion of p53 uncoupling from its negative regulators (MDM2, MDMX); *b)* effect on the interaction of p53 with p300/CBP transcriptional coactivator proteins and other binding partners; *c)* determination of which target genes would be induced following p53 activation and subsequent p53-mediated cell fate – e.g., cell cycle arrest or cell death; *d)* suppression of p53 activity; *e)* induction of conformational changes of p53 protein, etc. Although numerous studies during the last decades have significantly contributed to our knowledge of PTMs

of p53 and their impact on p53 function, there is still a long way to go in order to fully understand the role of p53 modifications in cellular processes.

Mutant p53. *TP53* alterations are common in tumor cells and mainly represent missense mutations, resulting in stably expressed protein with a single amino acid substitution. Most of these substitutions are found in the DNA-binding domain of p53, more than that – about one third of them are located in six "hotspot" sites: R175, G245, R248, R249, R273 and R282 [148]. According to their impact on thermodynamic stability of protein, p53 mutations are classified into DNA-contact and conformational mutations [136, 148, 149]. The mutations of the first category do not cause significant conformational changes in p53 protein, but rather occur in residues directly involved in contact with the backbone of DNA, therefore, weakening p53 DNA-binding activity hampering transactivation of target genes (for example, R273H and R273C p53 mutants). The second category, in turn, includes amino acid substitutions in the domains of p53 protein that are important for DNA binding. In contrast to the DNA-contact mutants, conformational mutations lead to adoption of non-native conformation of p53 protein, resulting in dramatic loss of DNA-binding activity (G245S and R249S). Mutant p53 proteins are frequently characterized by high stability that leads to their accumulation in the cell, in contrast to wtp53, which is maintained at low levels through MDM2 regulation under normal conditions [150]. In fact, positive immunohistochemical (IHC) staining of cells is considered an indicator, though not a proof, of accumulation of p53 with missense mutations.

Even in the case of complete loss of wild-type function, mutant p53 may be characterized by a dominant-negative inhibitory activity - heterooligomerization of mutp53 with wtp53 monomers results in formation of an inactive tetramer. What is more important, mutp53 proteins often not only lose normal tumor suppressor activity and serve as dominant-negative inhibitors of

wtp53, but also acquire novel oncogenic functions, and therefore are referred to as “gain of function” (GOF) mutants [151].

Although mutations have been reported for nearly every position in the DNA-binding domain of *TP53*, the frequency of specific missense mutations largely depends on the type of cancer and also the subclass of tumor of the same organ. However the most frequent p53 protein mutants are found in various cancer types and can be associated with poor prognosis and aggressive behavior of tumor cells [149].

Furthermore, the consequences of missense mutations of p53 are determined by the position of mutation as well as the type of substitution. In summary, oncogenic or gain-of-function properties acquired by mutp53 demonstrated in numerous studies may result in: a) genetic instability in tumor cells; b) increased cell proliferation and survival; c) promotion of angiogenesis, invasion and metastasis; d) resistance to chemo- and radiotherapy; e) prevention of apoptosis; f) maintenance of stemness (circumventing differentiation), etc. (Figure 1.7) [148, 152]. These are achieved through two main mechanisms, which are not mutually exclusive, such as mutp53 interaction with specific binding partners in the cell, and regulation of novel target genes by mutp53 [150].

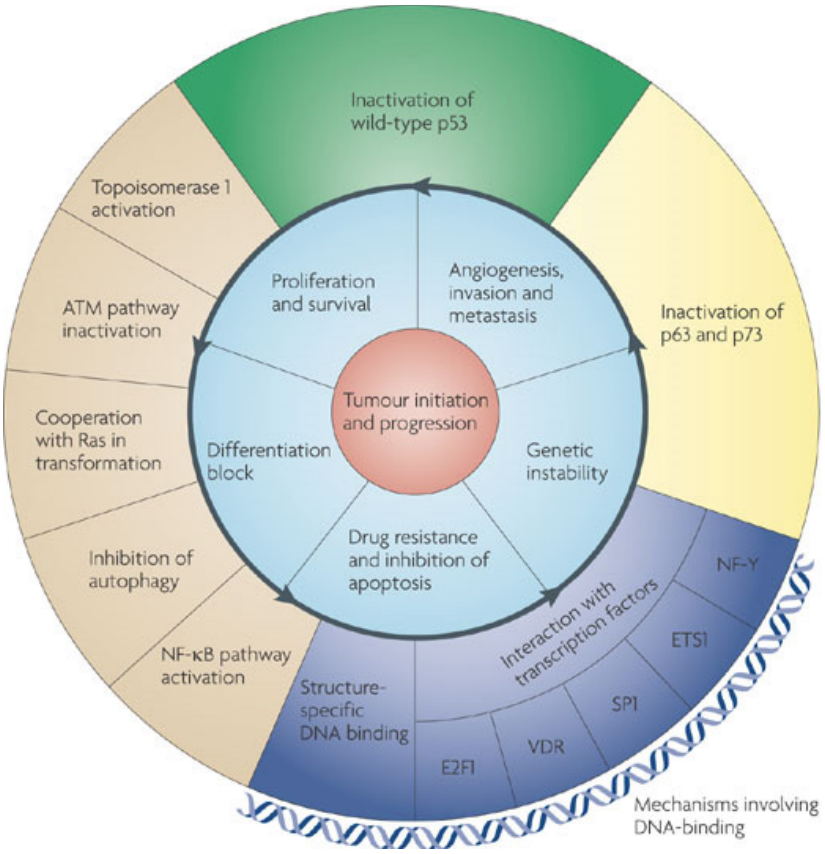

Figure 1.7. Oncogenic properties of mutant p53 (outer circle) that lead to oncogenic phenotypes (inner blue circle) (adapted from [148])

In terms of clinical importance, the predictive and prognostic value of *TP53* status is still debatable due to a number of reasons, such as the lack of standardized protocols for detection of p53 mutations in tumor samples (IHC, sequencing, etc.) and the site of malignancy. In fact, more than half of the studies of breast, colorectum, head and neck cancer and that of haematopoietic system showed that p53 mutations are associated with worse prognosis for patients' outcome, while in brain cancer many reports fail to demonstrate a clear association with outcome as such [148]. In addition, mutp53 may affect tumor response to therapy; therefore, restoration of wild-type functions of mutp53, which would be discussed in more details later, seems to be an attractive approach.

1.2.3 The role of p53 in GBM

The alterations in *TP53* gene are found in up to 30% of primary GBM tumors and in up to 60% of secondary GBM [153, 154], suggesting that p53 is important for tumor progression from low grade to high grade glioma (secondary GBM) and for progression of *de novo* high grade glioma (primary GBM). In particular, increased onset of *TP53* mutations has been reported in the "IDH", "K27", "G34" epigenetic subgroups of GBM with a "Proneural" expression signature [24, 26], described above. Numerous studies focused on p53 status as a prognostic biomarker for outcome of patients diagnosed with GBM as well as their response to radio-and chemotherapy. However, its role in GBM remains unclear due to contradictory results of these studies [155-159]. The results of studies showing that p53 mutations in GBM are favorable for response to therapy and longer survival could be explained by the failure of tumor cells possessing mutp53 to efficiently repair DNA damage caused by therapeutic agents, in contrast to cells with wtp53, leading to induction of their death through apoptosis. However, other studies show no association between p53 status and survival of GBM patients or association of p53 GOF mutations with poor prognosis [160]. The exact role of p53 mutations in GBM progression and response to therapy, therefore, remains to be clarified. Inconsistency in results of these studies may be due to a number of reasons, including the variety in study designs and methods [154], as well as failure of taking into account other factors, such as p53 isoforms and single-nucleotide polymorphisms, prognostic value of specific p53 mutations as well as concomitant alterations in p53 family members (p63, p73) and other signaling pathways.

1.2.4 Potential relationship between p53 and MGMT

Due to p53 activity as a transcription factor regulating expression of hundreds of genes and involvement of both p53 and MGMT in regulation of DNA repair, a number of studies have suggested that p53 may affect MGMT activity and expression *in vitro*. In particular, Harris et al. [161] showed that expression of exogenous wtp53 suppressed MGMT promoter activity in a reporter gene system in p53-null osteosarcoma cell line (also confirmed by Natsume et al. [122]) and inhibited expression of endogenous MGMT in human lung fibroblasts. Similar findings were reported by Srivenugopal et al. [162], showing that induction of exogenous or endogenous p53 expression in a panel of cancer cell lines, including gliomas and GBM cell lines, led to drastic reduction in MGMT mRNA, protein levels and activity, while making them more susceptible to alkylating agents. Further in a mechanistic study Bocangel et al. [163] demonstrated that ectopic expression of wild-type, but not mutant, p53 in a p53-null colon carcinoma cell line, transfected with MGMT-reporter plasmid, dramatically suppressed activity of MGMT promoter. By using the minimal MGMT promoter sequence required for reporter expression that contained three binding sites for *Sp1* transcription factor, the group showed that this region is sufficient for wtp53-mediated inhibition of MGMT. Furthermore, by showing that overexpression of *Sp1* relieved inhibition of MGMT mediated by p53 and that wtp53 can physically interact with *Sp1*, they elegantly demonstrated that p53-mediated repression might be achieved via preventing the *Sp1* from binding to the MGMT promoter. Recently, Kim et al. used the liposome-based nanodelivery system (scL-p53) carrying wt *TP53* gene (which will be discussed in more detail in the next subsection) to demonstrate that treatment with scL-p53 induced silencing of MGMT expression in MGMT-proficient glioblastoma cells *in vitro* and in mouse xenograft models of GBM tumors [164], which is in accordance with the hypothesis of negative MGMT regulation by wtp53.

In contrast, at least two studies suggested the opposite effect of wtp53 on MGMT levels. In particular, Grombacher et al. [165] showed that induction of MGMT promoter activity, mRNA and protein expression in various mouse fibroblasts and rat hepatoma cell lines upon exposure to IR was dependent on the presence of functional wtp53 and did not occur in the cells expressing mutp53. However, the overexpression of wtp53 by its transfection into the cells was associated with reduced basal activity of MGMT and inhibited its induction by IR. The authors explain this dual effect of p53 on MGMT promoter activity by the possibility that overexpression of transfected p53 might result in unphysiologically high levels of the protein, which do not reflect the normal conditions in the cell. Another group [166] found that wtp53 knockdown by RNAi led to significant downregulation of MGMT expression without affecting promoter methylation in human astrocytic glioma cells and in neonatal murine astrocytes, showing direct binding of p53 protein to its consensus site at the murine MGMT promoter. The mechanism of MGMT inhibition by p53 in human glioma cells remains unknown.

As mentioned above, Bobustuc et al. [127] showed that mutp53 was able to inhibit MGMT transcription in GBM cell lines treated with antiepileptic drug (levetiracetam), which promoted binding of mSin3A/HDAC1 transcription co-repressor complex and p53 to MGMT promoter, thus, suppressing MGMT expression.

In order to determine the relationship between MGMT and p53 in tumor cells numerous other works focused on assessing the correlation between MGMT promoter methylation/ expression and p53 status/ expression in tissue samples from different types of cancer:

a) Correlation between MGMT promoter methylation and G:C→A:T transition mutations in p53. Several studies suggested that *MGMT* promoter methylation, the main mechanism of

epigenetic MGMT silencing, is frequently associated with G:C→A:T transition mutations in p53. In particular, Nakamura et al. [167] found that p53 mutations were significantly more frequent in tumor tissue samples with MGMT promoter methylation obtained from patients with low grade gliomas - 92% (out of 26 cases) and secondary GBM - 92% (out of 12 cases), but not primary GBM – 8% (out of 13 cases). More specifically, G:C→A:T transition type of mutations was detected in 58% and 50% of low grade gliomas and secondary GBM with methylated MGMT promoter, respectively, but were not found in primary GBM with methylated MGMT. In their study Watanabe et al. [159] analyzed tumor tissue samples from patients diagnosed with anaplastic astrocytoma (AA) or GBM (45 cases) and detected 17 samples that were characterized with MGMT promoter methylation (7 AA and 10 GBM), of which 7 (41%) possessed *TP53* mutations, 57% of them G:C→A:T transitions. In contrast, only 18% of samples (out of 28) with unmethylated MGMT promoter possessed mutp53, only 1 of which was of G:C→A:T transition type. Analysis of samples of nervous system tumors (469 cases), including glioblastomas and anaplastic gliomas, by Bello et al. [168] showed that tumors with methylated MGMT promoter were significantly more enriched with p53 mutations in general (25%) compared to those with unmethylated MGMT (10%), and also had higher incidence of G:C → A:T transition mutations of *TP53* occurring at CpG sites or at non-CpG dinucleotides. Similar trend was reported for colorectal tumors (314 samples) [169], where methylated MGMT tumors were more frequently characterized by appearance of G:C to A:T transition mutations of *TP53* compared to unmethylated ones (34% compared to 19%), while the correlation was even stronger when taking into account G:C to A:T transitions only in non-CpG dinucleotides – 71% of all non-CpG transitions in p53 were found in methylated MGMT tumors. Finally, in lung tumors (220 cases) [170] MGMT promoter

methylation was more prevalent in samples with G:C to A:T transitions of p53 as well as other types of p53 mutations compared to tumors with wtp53.

G:C → A:T transitions that are mostly detected in GC-rich CpG sites can occur through several underlying mechanisms. The best known one is the spontaneous deamination of 5-methylcytosine to thymine, which can be caused by various factors, such as reactive oxygen species, nitric oxide, etc [167]. Another possible mechanism is the O6-methylguanine mispairing with thymine resulting from absence of MGMT DNA-repair activity due to silencing of MGMT expression by promoter methylation. However, the contribution of each mechanism into accumulation of G:C → A:T transitions in p53 is hard to determine, because of difficulty in distinguishing between these two mechanisms during analysis of tumor tissue samples.

b) Correlation between MGMT promoter methylation and TP53 status. A group of Shamsara et al. [171] found that positive IHC staining for p53, considered as an indicator of mutp53 status due to stabilization of mutant protein compared to short-lived wtp53 that allows its detection, was strongly associated with MGMT promoter methylation (65% of samples with mutp53) compared to unmethylated MGMT (34.6%), in the analyzed GBM tumors (50 cases). Using lung cancer cell models *in vitro*, Lai et al. [172] showed that knockdown of endogenous wtp53 led to increase in MGMT promoter methylation in these cell lines, which was associated and potentially promoted by increase in chromatin remodeling proteins - DNA-methyltransferase 1 (DNMT1) and HDAC1 that bound to the MGMT promoter. The transient expression of wtp53 in p53-null cell, in contrast, caused decrease in MGMT methylation and DNMT1 and HDAC1 levels, whereas expression of mutp53 did not have any effect. The authors suggest that MGMT methylation might be modulated by p53 status and further lead to accumulation of p53 mutations in the absence of MGMT activity in advanced lung tumors.

c) Correlation between MGMT expression and TP53 status. Analysis of MGMT mRNA levels using semi-quantitative RT-PCR and p53 status by IHC staining performed on 39 GBM tumors, including 3 recurrent tumors [173], showed that MGMT expression was significantly lower in GBM tumors with mutp53, which provides evidence for potential promotion of p53 alterations in the absence of MGMT protein activity. Similarly, using tumor samples from 50 GBM cases Lotfi et al. [174] found significant correlation between IHC-negative MGMT (low levels of expression) and IHC-positive p53 (*TP53* mutant) staining. The authors, thus, suggested a hypothesis that upregulation of wtp53 during early phase of GBM development causes downregulation of MGMT expression, which is suppressed even more as a result of MGMT promoter methylation during GBM progression. As previously mentioned, absence of MGMT activity may further lead to gradual accumulation of p53 mutations in GBM tumors. Another group analyzed samples from 35 cases of diffusely infiltrating astrocytomas using IHC [175] and found that the percentage of MGMT positive cells is significantly decreased in high grade compared to low grade astrocytomas, whereas MGMT negative tumor cells tended to have mutp53 (IHC-positive staining), which is in accordance with the above hypothesis of Lotfi et al. Importantly, similar results were obtained by the analysis of tumors from 48 primary breast cancer patients [176], which showed that tumor cells negative for MGMT (IHC-negative) had significantly higher expression of p53 (IHC-positive), indicating that the correlation between low MGMT expression and mutp53 might be true not only for glial tumors, but also for other types of cancer.

d) Correlation between MGMT activity and TP53 status. A number of studies assessed activity of MGMT protein in tumor tissue sections using a specific assay [177] and analyzed its relation to p53 status. Particularly, Wiewrodt et al. [178] showed that in tumor tissue sections obtained from 40 patients with primary GBM, there was a tendency for lower MGMT activity

when high percentage of cells positive for p53 (>10%) was detected by IHC (considered to be mutp53). Interestingly, analysis of tumor tissue samples collected from 159 patients with ovarian carcinoma (n=140 - primary cancer, n=19 – recurrent tumors) [179] showed that MGMT activity was highly variable in different patients and increased with progression from lower grade to higher grade. In contrast to the results in GBM tumors described above, ovarian carcinoma tumors with wtp53 status demonstrated lower MGMT activity than those with mutp53, whereas both MGMT activity and percentage of p53 mutant tumors increased with progression to higher grade.

In conclusion, the discrepancy in the results of multiple studies described in this section demonstrates the complexity of MGMT regulation and its relationship with p53. The conflicting data on MGMT:p53 association might be due to the differences in *in vitro* models used and the physiological relevance of manipulations of genes expression in them as well as the methods utilized to detect MGMT expression or methylation status and p53 status in tumor tissue sections and the type of cancer being investigated. Clearly, more investigation is needed to clarify the nature and magnitude of the association between MGMT and p53 in cancer cells and to understand molecular mechanism of this relationship.

1.3 STRATEGIES OF p53 TARGETING

Due to high incidence of disturbances in normal functioning of p53 or other proteins involved in p53 signaling in cancer, development of anti-cancer therapies targeting p53 pathway and their translation into clinic are of a great interest. This fact is supported by numerous completed and ongoing clinical trials assessing the efficacy and safety of multiple agents intended to restore p53 function in cancer cells [180]. Currently, the strategies employed in these studies can be classified into such major groups: *TP53*-based gene therapy, p53 vaccines, agents for prevention

or disruption of p53-MDM2 interaction, and small compounds for rescue of mutp53 (Table 1.1), and each of them will be discussed in more details below [180-182].

Table 1.1. Strategies of p53 targeting

Type of action	Compounds
TP53-based gene therapy	• Gendicine; • Advexin; • ONYX 015; • H101; • Nanoparticles
p53 vaccines	• p53 synthetic long peptide (p53-SLP); • dendritic cell-based p53 vaccine (Ad.p53-DC; INGN-225);
Agents for prevention or disruption of p53-MDM2/MDM4 interaction	• Antisense anti-MDM2 oligonucleotides (inhibit MDM2 expression); • HLI98 (inhibits E3 ubiquitin ligase activity of MDM2); • Nutlin-3a/RO5045337/RG7112, MI-219, ISA27, TDP665759 (block MDM2/p53 complex formation by binding to MDM2); • WK298/Novartis-101, SJ-172550, PMI (block MDMX(2)/p53 complex formation by binding to MDMX and to a lesser extent – to MDM2); • RITA (blocks MDM2/p53 complex formation by binding to p53; alternative mechanisms suggested); • JNJ-26854165/Serdemetan (activates p53 via unknown mechanism)
Mutant p53 reactivation compounds	• P53R3; • CP-31398; • RETRA • peptide 46; • CDB3; • PhiKan083 and PK7088 (target Y220C-p53 mutant) • SCH529074 • Ellipticine • WR1065 • MIRA-1 • STIMA-1 • PRIMA-1 and PRIMA-1^{MET}

1.3.1 *TP53*-based gene therapy

This strategy aims to restore wild-type function of p53 by introducing the complementary DNA copy of p53 gene into the cell with the help of the vectors: a) viral - usually of adenoviral origin (Adp53) [183] or b) plasmid – encapsulated into the nanoparticles, which do not require integration into the genome of the host cell for p53 expression.

A) Adenoviral p53 (Adp53) was shown to suppress cell proliferation, induce cell cycle arrest and massive apoptosis in a number of studies as well as radiosensitize GBM cells *in vitro* and also inhibit tumor growth *in vivo* [184-188]. Gendicine was the first Adp53 gene therapy product approved for clinical use in humans. This replication-incompetent recombinant adenovirus serotype 5 designed to express human wtp53, has been tested in multiple clinical trials for various cancers, including advanced laryngeal cancer, non-small-cell lung cancer, hepatocellular carcinoma, squamous cell carcinoma of head and neck, etc. either as a single agent or in combination with chemo- or radiotherapy [183], which resulted in approval of this drug by the China State Food & Drug Administration in 2003 for treatment of head and neck squamous cell carcinoma [189].

Another adenoviral vector carrying wtp53, Ad5CMV-p53 (Advexin) was tested in phase I and II clinical trials for patients with chemoradiation-resistant advanced esophageal squamous cell carcinoma or squamous cell carcinoma of the oral cavity, oropharynx, larynx, or hypopharynx [190, 191]. Although they showed safety, feasibility and biologically activity (induction of p21, etc.) of the drug injected either in an intratumoral or perioperative manner to the primary tumor bed, additional phase III clinical trials are still needed to provide sufficient data for its approval by the Food and Drug Administration.

Another approach is to utilize adenoviruses with deletion of a gene encoding E1B-55kD, a protein that would normally bind and inhibit wild-type p53 in the host cell in order to achieve efficient virus replication. E1B-55kD-deleted adenovirus is not capable of degrading p53 and its replication is, therefore, blocked in only in wtp53 expressing cells, while mutp53 tumor cells are destroyed as a result of productive virus infection. One of the most studied representative of this group is ONYX 015 vector [192], which presumably replicates only in cancer cells possessing mutp53, although some reports showed p53 status-independent activity of this vector, suggesting that ONYX 015 selectivity most probably depends on the state of the stress response in the host cell [193, 194]. The phase I clinical trial assessing safety and the efficacy of ONYX 015 introduced through multiple injections of escalating doses into sites within the resected tumor cavity in adult patients with recurrent malignant glioma showed that this agent was well tolerated, however no definite anti-tumor efficacy was demonstrated [195]. Moreover, despite the fact that this vector has been tested in numerous clinical trials for other cancer types, such as squamous cell carcinoma of the head and neck, refractory metastatic colorectal cancer, unresectable pancreatic carcinoma, advanced sarcomas, etc., its approval for use in clinics did not occur due to very high variability in clinical efficacy of this drug and unclear mechanism of action.

H101, an adenoviral vector very similar to ONYX 015, has been tested in phase II clinical trial for late stage cancer patients with more than two measurable lesions in People's Republic of China [196]. The reported response rate of tumors that were repetitively injected with H101 was significantly higher than that of control lesions, indicating anticancer activity of the vector, whereas no severe toxicity was detected. A subsequent phase III trial enrolled 160 patients with head and neck or esophagus squamous cell cancer and assessed the efficacy of intratumoral H101 injection combined with cisplatin plus 5-fluorouracil (PF) regimen compared to PF alone [197].

The results showed that the response rate was significantly better for combination (78.8%) compared to chemotherapy alone (39.6%), while H101 was well-tolerated. As a result, the China State Food & Drug Administration approved H101 to be used in combination with chemotherapy as a treatment for patients with head and neck cancer in 2005 [198].

Despite promising data obtained *in vitro* and in clinical trials, there are limitations to the adenovirus-based approach use for cancer in general and for GBM, in particular, which include the variability in effectiveness of gene transfer to host cells (inability to infect every tumor cell), the problems with selective infection of tumor cells (but not normal cells) and the potential development of resistance due to extreme heterogeneity of GBM [183, 199], which however might be prevented by utilizing different combination regimens, for example combining Adp53 with radiation.

B) *Nanoparticle-mediated gene delivery* is an emerging strategy, in which DNA-containing plasmids are encapsulated within non-viral systems, such as liposomes formed from cationic lipids, cationic polymers and polymeric vesicles or a combination of cationic lipids and polymers complexed with DNA [200]. Although demonstrating lower efficiency than viral vectors, non-viral systems are raising less concerns regarding safety for patients compared to the first ones. There were a number of studies aiming to design and develop the efficient delivery of non-viral vectors carrying wt*TP53* gene alone or combined with chemotherapeutic agents, to different types of tumors, some of which are described below.

The tumor targeting nanocomplex TfRscFv/Liposome/p53 (scL-p53 or SGT-53) mentioned in the previous subsection, was developed by Kim et al. and consisted of the wt*TP53* gene containing plasmid encapsulated into a cationic liposome (scL) with an anti-transferrin receptor

(TfR) single-chain antibody fragment (scFv). The function of TfRscFv is to provide selectivity towards the tumor cells through specific binding to TfR, known to be expressed at high level by many tumor cells on their surface, and to provide internalization of the nanocomplex via the receptor-mediated endocytosis. In addition, due to expression of TfR on the surface of cerebral endothelium of the blood-brain barrier (BBB), scL was shown to efficiently cross the BBB via the transcytosis mediated by TfR, a feature that is critical for application in GBM. When tested in GBM, scL-p53 was able to inhibit MGMT expression in MGMT-proficient GBM cells *in vitro* and in mouse xenograft GBM model [164]. Moreover, treatment with scL-p53 sensitized TMZ-responsive and TMZ-resistant GBM cells to treatment with this alkylating agent *in vitro* and *in vivo* [201]. Importantly, scL-p53 was well-tolerated by patients with advanced solid tumors and demonstrated targeted tumor delivery in phase I clinical trial [202]. Therefore, addition of scL-p53 could potentially sensitize GBM tumors to the standard therapy and improve GBM patients' response and survival, while decreasing the frequency of adverse effects by allowing to use lower doses of conventional chemo- and radiotherapy.

In their study, Prabha et al. engineered nanoparticles, formulated using biodegradable polymers, poly(d,l-lactide-co-glycolide) (PLGA) and polylactide (PLA), and loaded with *TP53* gene-containing plasmid (p53NPs) [203]. The p53NPs showed sustained release of p53 DNA associated with stable expression of p53 in tumor cells following administration in p53-*null* (prostate cancer) or mutp53 expressing (carcinoma) mouse models. Treatment with p53NPs led to inhibition of tumor growth and angiogenesis as well as increased animal survival compared to control.

Other p53-containing nanoparticles were tested in hepatocellular carcinoma (double-walled polymeric microspheres) [204], pancreatic cancer (gelatin nanoparticles) [205], small cell lung

cancer (biodegradable poly(β-amino ester) (PBAE) polymeric nanoparticles) [206]. Their ability to cross BBB remains to be elucidated.

1.3.2 p53 vaccines

The strategies of p53 vaccines development are based on the finding that cancer patients, whose tumor cells are characterized by high levels of p53, often produce antibodies to p53 [207, 208], while they are rarely found in cancer patients with tumors expressing low levels of p53 or in healthy individuals. More than that, in the study by Met et al., 40% of breast cancer patients were shown to have pre-existent T-cell reactivity against p53 at primary diagnosis [209]. Therefore, it was hypothesized that p53 might be used as a target tumor-associated antigen for immune therapy aiming to induce strong immune response against tumor cells. Since then a number of clinical trials were conducted to assess safety and efficacy of p53 vaccines. In particular, patients with metastatic colorectal cancer enrolled in phase I/II study were subcutaneously vaccinated with p53 synthetic long peptide (p53-SLP) vaccine consisting of 10 overlapping peptides, together corresponding to amino acids 70-248 of the p53 protein, which represent it most immunogenic part [210]. The results demonstrated favorable toxicity profile of p53-SLP and induction of p53-specific T-cell responses in most of the patients, while in more than half of them it persisted for at least 6 months. However, there was a low production of proinflammatory cytokines by induced T-cells, therefore a prolonged vaccination scheme might be required in order to achieve a stronger polarized T-cell response.

In another phase II trial [211] the peripheral blood mononuclear cells (PBMC) were collected from patients with extensive-stage small cell lung cancer and following a series of preparation steps the population of dendritic cells (DCs) was recovered. Then DCs were transduced with an adenoviral construct containing wtp53, Advexin, described above, to produce a dendritic cell-

based p53 vaccine (Ad.p53-DC; INGN-225). Finally, patients from which PBMC were obtained, repeatedly received INGN-225 intradermally and safety and efficacy of the therapy was assessed. INGN-225 was found to be well-tolerated independently of the dose (number of $p53^+$ DCs) used and capable to induce significant anti-p53 immune response (41.8% of patients). In addition, in patients, who developed positive immune response, INGN-225 seemed to sensitize tumors to second-line chemotherapy, resulting in better clinical response.

Although, to our knowledge, no clinical data was reported on the use of p53-based vaccines in GBM, a number of trials were conducted assessing the potential of immunotherapy in this type of cancer by targeting other tumor-associated antigens. In particular, EGF receptor (EGFR) variant (EGFRvIII) - the most common variant of the EGFR in GBM - has been targeted with the vaccine containing a 13 amino acid sequence unique to EGFRvIII (CDX-110) given intradermally to adult patients with newly diagnosed EGFRvIII-expressing GBM in several phase II clinical trials. In the trial that is currently in progress testing the combination CDX-110 with standard radiotherapy and TMZ [212], the interim analysis of the first 40 patients demonstrated that the vaccine was well-tolerated and 70% of patients were progression free at 5.5 months, similar to the results of another completed trial (PFS=67% at 6 months) [213]. Furthermore, other tumor-associated antigens are being targeted using several different strategies: *i)* direct introduction of these peptides (coadministration with an adjuvant); *ii)* use of DCs preloaded with antigen; *iii)* administration of heat shock proteins bound to tumor peptides (HSP-peptide complex).

Based on the encouraging data from these and other studies, immunotherapy seems to be a promising strategy for treatment in GBM allowing to selectively target tumor cells based on their specific antigens or even certain tumor cell populations without damaging normal tissue. At the same time the use of such an approach in GBM needs to deal with and overcome such challenges

as the protumorigenic and immunosuppressing properties of the GBM microenvironment (expression of immunosuppressive cytokines, increased population of regulatory T cells, etc.) [214], as well as inhibition of immune function by radiation therapy, temozolomide and steroids [215, 216].

1.3.3 Agents for prevention or disruption of p53-MDM2/MDM4 interaction

The compounds for blocking of p53-MDM2/MDM4 interaction are developed based on the knowledge that E3 ubiquitin ligase MDM2 is the primary negative regulator of p53 (as discussed earlier), targeting it for ubiquitin-dependent proteosomal degradation. In addition, it is a transcriptional target of p53, thus, forming a negative feedback loop for tight control of p53 levels in the cell. MDM4 (MDMX) protein is not able to ubiquitinate p53, but can form heterodimers with MDM2, therefore, promoting p53 degradation. Importantly, elevated levels of MDM2 through gene amplification or protein overexpression as well as silencing of MDM2 inhibitors ($p14^{ARF}$) are frequently found in cancer allowing the tumor cells to achieve inadequate p53 function even in the context of wtp53 through its downregulation and subsequent lack of activation of p53-mediated downstream signaling [180]. Therefore, disruption of p53-MDM2 complex and/or inhibition of MDM2 in order to reactivate p53 and potentially promote apoptosis of tumor cells, seem to be a promising approach for cancers possessing wt*TP53*. Still one has to take into account the possible difficulties for successful use of this strategy in the clinic, such as the possibility of wtp53 inhibition by other mechanisms in addition to MDM2 (ARF-BP1, PIRH2 proteins, etc. [217]), aberrant signaling downstream of the p53 pathway, potential cytotoxicity for normal cells [218]. Several strategies have been employed to prevent MDM2-mdeiated suppression of p53 in tumor cells.

A) Inhibition of MDM2 expression by antisense anti-MDM2 oligonucleotides resulted in elevated p53 levels in cell lines of different types of cancer, including colon, lung, breast, and prostate and GBM [219], and also decreased tumor growth in mouse models [220]. The antisense oligos even caused upregulation of p21 protein, but not p53, in human cancer cell lines with mutp53, however more investigation is needed in order to explain this observation.

B) The discovery and development of *inhibitors of E3 ubiquitin ligase activity of MDM2* should allow preventing ubiquitination and degradation of p53 and, instead, promoting its stabilization as well as activation of p53-dependent transcription and apoptosis. Particularly, a high-throughput screening of libraries of small molecules capable to suppress MDM2 autoubiquitylation by more than 50% conducted by Yang et al. [221] resulted in the identification of a family of closely related compounds, HLI98, which significantly inhibited p53 ubiquitylation *in vitro* at doses of 20-50 μM. In addition, HLI98 caused an increase in p53 and MDM2 (as a result of inhibition of autoubiquitylation) expression as well as p21 in primary human fibroblasts. However, these compounds were also shown to inhibit other E3 ligases, although to a less extent than MDM2, suggesting the lack of sufficient selectivity of these small molecules and the need for their further assessment both *in vitro* and *in vivo*. The recent report on the synthesis of new analogs of HLI98, which show higher activity and selectivity towards MDM2 [222], holds promise that an approach of targeting the E3 ligase activity of MDM2 could be developed further and this class of small molecules could potentially be used for p53 activation in tumor cells.

C) Another approach is to *inhibit the formation of the MDM2(MDMX)-p53 complex* by preventing the interaction between two proteins. Importantly, the finding that only three amino acids of p53, Phe19, Trp23 and Leu26, are crucial for MDM2 binding [223] gave a boost for the development of small molecules mimicking the interaction of p53 with a hydrophobic pocket at

the N-terminus of MDM2 molecule (p53 pocket). In particular, a class of cis-imidazoline analogs capable of inhibiting p53-MDM2 complex at nanomolar range of concentrations, was identified by Vassilev et al. [224] during the screening of chemical library, and these compounds were named Nutlins (for Nutley inhibitor). In fact, the analysis of crystal structure of nutlin-MDM2 complex showed that nutlins indeed occupy the MDM2 pocket, which is normally bound to three amino acids of p53. In accordance with negative regulation of p53 by MDM2, treatment with nutlins induced upregulation of p53 and its targets, p21 and MDM2, in human colon cancer cell line with wtp53 indicating that p53 got released from MDM2-mediated suppression and restored its transcriptional activity. In addition, nutlins caused G1 and G2/M cell cycle arrest, inhibited proliferation and subsequently led to apoptosis of human osteosarcoma and colon cancer cells *in vitro*, whereas *in vivo* nutlins were able to inhibit tumor growth (human osteosarcoma xenograft) by 90% compared to control.

Further the most potent among nutlins, nutlin-3a, underwent several modifications to improve its affinity to MDM2 as well as other physicochemical properties and resulted in a synthesis of the compound RO5045337 (RG7112) [225, 226], which became the first small MDM2 antagonist to enter clinical trial. Particularly, in patients with well-differentiated or dedifferentiated liposarcoma possessing *TP53* wild-type tumors and, in majority, amplification of *MDM2*, treatment with RG7112 resulted in increased expression of p53, p21 and MDM2 as assessed by IHC [227], indicating that this compound was able to reactivate p53 pathway in the tumor cells. Two other phase I trials assessing maximum tolerated dose and optimal dosing schedules of this compound in patients with advanced solid tumors or hematologic neoplasms (clinicaltrials.gov identifiers: NCT00559533 and NCT00623870) have been completed and the results are to be released.

The in-depth analysis of the structure of the MDM2-p53 complex previously determined by X-ray crystallography allowed Ding et al. [228] to assume that Trp23 amino acid of p53 is the most critical for binding to MDM2. Furthermore, their finding that oxindole can mimic the indole ring of Trp23 led to the structure-based rational design of a number of non-peptide compounds (spiro-oxindoles) capable of binding MDM2. Following subsequent optimization of these compounds [229] they demonstrated significant increase in affinity to MDM2, while also showing good selectivity between cancer and normal cells with wtp53 *in vitro* (human prostate cancer cell lines), although they were still less potent than nutlins. Additional modifications aiming to improve the affinity to MDM2, for example, introduction of chemical groups that mimic Leu22 and Glu17 of p53, led to more tight binding of the newly synthesized compounds to MDM2 and a potency of compound named MI-63 (MDM2 inhibitor 63) that was 12-times higher (3 nM) in comparison with Nutlin-3 (36 nM). In addition, MI-63 was shown to upregulate p53, MDM2 and p21 expression in human prostate adenocarcinoma cell line possessing wtp53. Further modifications of MI-63 were performed in order to improve its pharmacokinetic profile and resulted in a synthesis of MI-219 MDM2 inhibitor, which was able to mimic the interaction of Phe-19, Leu-22, Trp-23 and Leu-26 residues of p53 with MDM2 with high potency (5 nM) and also induced accumulation of p53 and increased levels of its targets - p21, MDM2 and PUMA, in osteosarcoma and prostate cancer cell lines with wtp53, but not with mutant or deleted p53 [230]. Interestingly, although upregulation of p53 targets as well as cell cycle arrest was detected in both cancer and normal cell lines upon treatment with MI-219, the induction of cell death through apoptosis occurred only in cancer, but not in normal, cells, suggesting selective activity of this compound. Importantly, MI-219 was shown to activate p53, upregulate its targets and induce apoptosis of tumor cells in xenograft cancer models *in vivo*, while also inhibiting tumor growth. Another study

recently showed that MI-219 enhanced MDM2 autoubiquitination and degradation in non-Hodgkin's lymphoma cell lines with wtp53, when compared to the effect of nutlin-3 [231], although further investigation is needed to understand the mechanism of this phenomenon. No clinical data is currently available for this compound.

Recently, a new MDM2 inhibitor with a spirooxoindolepyrrolidine core structure, ISA27, was synthesized and tested in GBM cells *in vitro* and in human GBM xenograft [232]. Particularly, it induced activation of p53 with subsequent cell cycle arrest and inhibition of GBM cell proliferation, resulting in apoptosis *in vitro* and *in vivo*, while showing no sign of toxicity to normal cells. Furthermore, a synergistic effect was observed when a combination of ISA27 and AKT/mTOR inhibitor was used in GBM cell lines and glioma stem cells with wtp53 [233]. The ability of this compound to penetrate the BBB remains to be determined.

The members of another class of small-molecule inhibitors, benzodiazepines (BDP), were identified during the screening of compound libraries using the ThermoFluor microcalorimetry based on detection of the shifts in thermal stability of target proteins upon binding by the compound [234]. One of the compounds, TDP665759 demonstrated antiproliferative properties towards cells with wtp53 *in vitro* and only a modest effect on growth of the A375 melanoma xenograft model, but led to significant tumor growth inhibition when combined with doxorubicin [235], suggesting synergy between this p53 stabilizing compound and DNA-damaging chemotherapeutic agent, doxorubicin.

Recently, Popowicz et al. reported the first co-crystal structure of small molecule bound to MDMX (MDM4) [236]. This compound, named WK298 or Novartis-101, was able to bind not only MDM2, but also MDMX with moderate affinity, in contrast to nutlin-3 and other tested

MDM2 inhibitors, which had much higher selectivity towards MDM2. The analysis of WK298-MDMX complex structure demonstrated that WK298 mimics three p53 residues, Phe-19, Trp-23 and Leu-26, important for interaction with MDM2 and MDMX. However, the activity of this compound in the cells still has to be characterized.

Another MDMX inhibitor, SJ-172550, was identified by high throughput screening of chemical library using fluorescence polarization assay and cell-based assay in retinoblastoma cells [237]. In addition to good chemical profile and stability in solution, SJ-172550 was also shown to bind the p53-binding pocket of MDMX in a reversible manner. Treatment of retinoblastoma and leukemia cells, possessing wtp53, with this compound induced accumulation of p53, although to a lesser extent compared to nutlin-3, as well as upregulation of p53 targets and cell death through apoptosis. Yet the efficacy of SJ-172550-mediated disruption of p53-MDMX complex and the potency of its use in combination with MDM2 inhibitors in different cancer models remains to be elucidated.

In contrast to compounds described above that mimic p53 binding to MDM2, a small molecule, RITA (reactivation of p53 and induction of tumor cell apoptosis) identified during the screening of the National Cancer Institute (NCI) library of compounds, inhibits p53-MDM2 interaction in a p53-dependent manner [238]. In this study RITA was shown to bind the N-terminal of the p53 protein using the fluorescence correlation spectroscopy and stabilize it leading to its accumulation in the cell and subsequent upregulation of p53 target genes (*CDKN1A*, *MDM2*) followed by apoptosis. RITA much more effectively suppressed growth of a panel of cancer cell lines with wtp53, than of those with mutp53 or p53-*null*, with similar results observed *in vivo*. The authors hypothesized that this compound blocks p53-MDM2 interaction by causing the changes in conformation of p53 protein and, thus, preventing MDM2 binding. Interestingly, in one of the

studies that followed, the RITA-induced p53-mediated transcriptional response (microarray analysis) and biological response associated with it (analysis of cell cycle progression) were distinct from the ones induced by another inhibitor of p53-MDM2 interaction – nutlin-3a [239]. In particular, in contrast to RITA that predominantly induced apoptosis in a panel of human cancer cell lines, response to nutlin-3a mostly manifested in a growth arrest in accordance with detected upregulation of p53 target genes involved in cell cycle regulation. More specifically, it was found that MDM2 released from the complex with p53 following RITA treatment bound to one of its other substrates – the transcriptional cofactor and coactivator for p53-induced growth arrest hnRNP K and promoted its proteosome degradation, while nutlin-3a did not affect hnRNP K levels in the treated cells. Importantly, expression of *CDKN1A* gene encoding p21 protein, the major target of hnRNP K and p53, was only transiently induced by RITA (first 4 hours), but kept increasing up to 8 hours following exposure to nutlin-3a. Mechanistically, although there was no difference in p53 recruitment to the *CDKN1A* promoter upon treatment with either RITA or nutlin-3a, hnRNP K recruitment to the site upstream of the promoter was increased following treatment with nutlin-3a, but not with RITA. These findings prompted authors to suggest that RITA induced MDM2-mediated downregulation hnRNP K, which was responsible for the absence of steady p21 upregulation and led to induction of apoptosis, rather than cell cycle arrest, as in the case of nutlin-3a. Furthermore, it was demonstrated that upon RITA-mediated release from the complex with p53 MDM2 itself promoted proteasomal degradation of the p21, thus, preventing cell cycle arrest. In contrast, upon nutlin-3a treatment MDM2 played a different role – it caused a degradation of HIPK2 kinase [240, 241], required for p53 phosphorylation at Ser46, a posttranslational modification of p53 usually detected after severe DNA damage that leads to apoptosis. Therefore, the biological response to the inhibitors of p53-MDM2 complex depends not only on activation of

p53, but also on the mode of MDM2 regulation of its other substrates, although the understanding of how and why certain substrates are chosen in every particular condition is lacking.

Moreover, even the original concept that RITA disrupts p53-MDM2 complex has been questioned [242]. RITA induced phosphorylation of wtp53 at Ser15 and of γH2AX at Ser139, indicating activation of DNA damage response, and also blocked HIF-1α and VEGF expression both *in vitro* and *in vivo*, without detectable blocking of p53-MDM2 interaction [243], suggesting that there are alternative mechanisms of RITA action. Further, the group that identified RITA, analyzed a panel of human cancer cell lines and reported that RITA is capable of suppressing the growth not only of wtp53-expressing cells, but also of cells carrying p53 with various mutations [244, 245]. In addition, it induced cell apoptosis in a mutant-p53 dependent manner and restored transcriptional activity to p53 mutants as demonstrated by induction of several p53 target genes. RITA also led to repression of a number of pro-survival genes and their protein products, such as c-Myc and Mcl-2, in cancer cells with wt or mutp53 [244, 246, 247]. Taken together, the mechanisms and effects of RITA-mediated reactivation of p53 seem far more complex than originally envisioned and require further investigation.

The tryptamine derivative, JNJ-26854165 (Serdemetan), is a p53-activating agent with unknown mechanism of action [248]. It demonstrated antiproliferative activity in acute leukemia cells with wtp53 *in vitro*, inducing p53 translocation to mitochondria and cell apoptosis [249]. Interestingly, although JNJ-26854165 induced p53-mediated activation of *CDKN1A* transcription, degradation of p21 protein encoded by this gene was enhanced through activation of proteasomes following the treatment. Induction of apoptosis by JNJ-26854165 was also detected in leukemia cells possessing mutp53, although through a different mechanism. JNJ-26854165 was shown to upregulate transcription factor E2F1 leading to E2F1-mediated apoptosis in S-phase,

independently of functional p53 and MDM2. Furthermore, when tested in cell lines derived from solid tumors, in particular, human non-small-cell lung carcinoma and colorectal cancer, this compound effectively suppressed cell proliferation and clonogenic survival of cells possessing wtp53, and to a lesser extent – p53-*null* cells [250]. In addition, pretreatment of wtp53 cells with JNJ-26854165 and further exposure to IR resulted in a significant increase in the level of clonogenic cell death, whereas *in vivo* combination of JNJ-26854165 and IR led to significantly longer tumor growth delay, compared to each of the treatments alone, thus, demonstrating the potency of JNJ-26854165 as a radiosensitizer. Indeed, JNJ-26854165 became one of few p53-activating compounds tested clinically, particularly its safety as well as pharmacokinetic and pharmacodynamic profiles were determined in the phase I clinical trial in patients with advanced, refractory solid malignancies [251]. In this study the drug showed dose-proportional pharmacokinetics with modest clinical activity, resulting from induced p53 expression in tumors. It was also well tolerated, although the observed exposure-related QTc prolongation (grade 2 and 3) raised a concern for its further testing in phase II trial.

Despite the growing number of studies reporting successful use of various MDM2 inhibitors in different cancer models, there are still some limitations to this approach. First, there is a possibility that p53 activation resulting from blocking p53-MDM2 complex upon administration of MDM2 inhibitor could be toxic to normal tissue, which needs to be taken into account [218]. Second, continuous use of MDM2 inhibitors might lead to acquired resistance of tumor cells to p53 activation. The resistance is also an issue for wt*TP53* gene therapy (Adp53), where activity of MDM2 as the major negative regulator of p53 may nullify the effects of transfected *TP53* expression [183]. Therefore, a strategy of combining Adp53 therapy with inhibitors of MDM2 E3 ligase activity or of formation of p53-MDM2 complex seems promising and needs to be

investigated as it could potentially assist in stabilizing both exogenous and endogenous wtp53 levels and increase the therapeutic efficacy of combined treatment. The third limitation for application of various MDM2 inhibitors is the necessity to be able to identify tumors/ tumor cells with downstream p53 signaling that is functional and could be activated upon stabilization of p53. Fourth, in GBM the efficacy of chemotherapeutic agents in general is limited, among others, by the integrity of the BBB preventing them from reaching the tumor site, and inhibitors of MDM2/MDMX are not an exception. Moreover, such factors as solubility, *in vivo* stability, tumor specificity and the levels of cellular uptake are major obstacles for development and introduction of new compounds for GBM treatment. However, there have been attempts to overcome these issues. For instance, Chen and colleagues [252] used a peptide inhibitor of the p53-MDM2/MDMX interactions, named PMI [253], and modified it by adding the all-hydrocarbon cross link, thus, creating a "stapled peptide" sPMI, characterized by increased protease resistance and cellular uptake [254]. Further, they developed a polymeric micelle (RGD-M) composed of cyclic RGD peptide–poly(ethylene glycol)–poly (lactic acid) (c(RGDyK)–PEG–PLA), which contains RGD peptide that recognizes αvβ3 and αvβ5 integrins overexpressed on glioma cells and neovasculature, thus, enabling targeted delivery to GBM tumor cells. sPMI peptide was loaded into RGD-M carrier (RGD-M/sPMI) and tested *in vitro*, inducing accumulation of p53 in treated U87MG GBM cells and increased levels of its targets – p21 and MDM2, leading to inhibition of proliferation, cell cycle arrest and apoptosis. *In vivo* RGD-M/sPMI significantly inhibited the growth of subcutaneous glioma tumor, when used alone and even more effectively in combination with TMZ. In addition, RGD-M/sPMI led to prolonged survival of mice bearing intracranial U87MG glioma and showed synergistic effect when used with TMZ. This study provides additional piece of

evidence supporting the rational for the use of wtp53 reactivating agents in combination with standard treatment for GBM.

1.3.4 Mutant p53 reactivation compounds

Mutations in *TP53* gene are found with high incidence in different types of cancer, such as breast, head and neck, ovarian [148, 255], and GBM (25-30% of IDH wild-type and about 80% of IDH mutant GBM) [154]. As discussed earlier, *TP53* alterations often lead to the acquisition of novel oncogenic GOF properties by mutp53 protein, which provides a rational for development of therapeutic agents targeting p53 mutants. Moreover, the fact that stabilized mutp53 protein in tumor cells is maintained at higher levels, compared to short-lived wtp53, should allow selective targeting of tumor cells without causing toxicity to normal cells. Over the last decades a number of compounds were identified and their ability to restore wtp53 function of mutp53 was tested *in vitro* and *in vivo*. Some of these molecules are reviewed below (Figure 1.8).

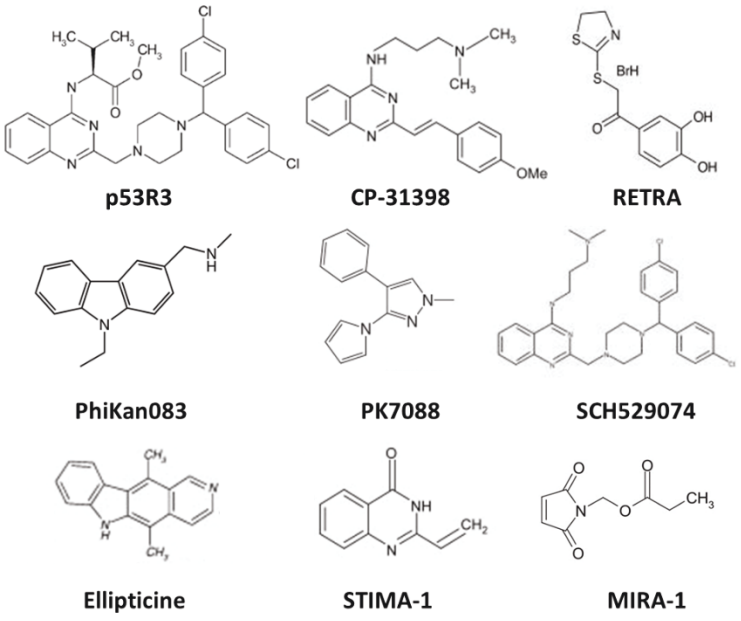

Figure 1.8. Structural formulas of mutant p53 reactivation compounds [256-263]

A quinazoline compound named P53R3 (p53 reactivator), identified by high-throughput screening of chemical library, is, to our knowledge, one of few mutp53 reactivation molecules (CP-31398, PRIMA-1) that were tested in glioma cells [258]. P53R3 suppressed proliferation of transfected p53-*null* human glioma cells expressing different exogenous p53 mutants and induced G0/G1 cell cycle arrest, which was not observed in control p53-deficient glioma or non-neoplastic cells. Furthermore, P53R3 increased the recruitment of mutant and, to a lesser extent, wtp53 to promoters of its target genes and led to upregulation of their mRNA expression (p21, PUMA, GADD45, etc.). In addition, P53R3 induced a p53-dependent increase in expression of death receptor 5 (DR5) protein and its translocation to the cell surface (p53-independent) in a panel of glioma cell lines and sensitized p53-expressing cells to Apo2L.0-induced apoptosis, suggesting synergistic effect.

Another compound, CP-31398, identified upon screening of a library of synthetic compounds was shown to stabilize newly synthesized mutp53 in the active conformation and inhibit growth of mutp53 tumor xenografts (melanoma, colon carcinoma cell lines) in a mouse model [264]. Further, CP-31398 tested in a panel of glioma cell lines induced loss of G2/M phase and accumulation of cells in sub-G0/G1 population, indicating cell death, disregarding of their p53 status (wt or mut) [265]. In addition, short treatment (1-4 hours) resulted in p53 activation and cytotoxicity exclusively in p53-expressing cells, whereas cytotoxic effects in p53-*null* glioma cell line were detected only following the long-term exposure to the compound, suggesting the presence of p53-independent mechanisms. Finally, the authors reported that the type of p53 mutation in glioma cell lines allowed predicting the ability of CP-31398 to enhance p53 activity in a reporter assay, however the observed increase in p53 activity did not necessarily coincide with cell line sensitivity to the drug or CP-31398-induced p53 stabilization and accumulation in the

cell. Thus, a clear understanding of the factors predisposing cells to cell death following exposure to CP-31398 is missing. Importantly, the combination of CP-31398 with adriamycin or cisplatin showed synergistic effect in colon and lung cancer cells [266], indicating the potential of restoring wild-type function to mutp53 prior to exposure of tumor cells to chemotherapeutic agents in order to activate the p53-mediated response to DNA damage and induce apoptosis.

Using a similar approach of high-throughput screening of a chemical library Kravchenko et al. [259] identified a compound that demonstrated activity in epidermal carcinoma cells expressing mutant (R273H), but not wtp53, and named it RETRA (reactivation of transcriptional reporter activity). RETRA (2-(4,5-dihydro-1,3-thiazol-2-ylthio)-1-(3,4-dihydroxyphenyl)ethanone hydrobromide) induced increased expression of such p53 targets as p21 and PUMA. However, it was further found that effect of RETRA was mediated through p53 family member – p73, which inhibition by shRNA led to substantial decrease in response to RETRA. Particularly, RETRA induced release of p73 from the complex with mutp53 protein, resulting in increased levels of active p73 in the cell. RETRA also showed the antitumor potential, when tested in mouse xenograft model inoculated with cancer cells expressing R273H-p53 mutant. The p73-dependent effects of RETRA – upregulation of p21, inhibition of proliferation as well as increase in caspase 3/7 activity and PARP-1 cleavage followed by apoptosis, were also reported for Kaposi's Sarcoma Herpesvirus-associated primary effusion lymphoma cells possessing mutp53, but not wtp53 bearing cells [267]. More recently, however, RETRA effects that are independent of *TP53* status were reported in Ewing's sarcoma (ES) cells [268]. In particular, this compound induced expression of PUMA and p21, G2/M cell cycle arrest, increased levels of cleaved form of PARP-1 and apoptosis in both p53-null ES cells and cells possessing either mutant (C176F, R273C, R273H) or wtp53. Thus, the possibility that the factors involved in RETRA-induced cytotoxicity

might differ in tumor cells of different origin should be taken into consideration during future preclinical and potential clinical assessments of this compound.

Based on the knowledge that the C-terminal domain of p53 protein allosterically regulates specific p53 binding to DNA and that small peptides corresponding to C-terminal residues of p53 are able to activate DNA binding by p53, Selivanova and colleagues [269] used peptide 46, corresponding to p53 residues 361-382, and showed that it caused increased activity of the p53-responsive reporter construct in mutp53 expressing human colon carcinoma cells and inhibited growth of osteosarcoma cells transfected with mutp53, leading to their apoptosis *in vitro*. The peptide 46-induced apoptosis was also observed in Burkitt lymphoma cells possessing wtp53, but not in p53-*null* cancer cell lines. The authors suggested a possible mechanism according to which the interaction between the C-terminal domain and the central core of p53 that usually locks wtp53 protein in its latent state is competitively disrupted by an excess of the C-terminal peptides, which bind to the p53 central core and release of the C-terminal domain, thus causing a conformational change in wtp53 protein leading to its activation. In the case of mutp53 protein, it was suggested that upon binding to the central core of p53 the C-terminal peptide stabilizes its interaction with DNA and, therefore, enhances mutp53 DNA binding capacity [270].

Another p53-binding peptide, CDB3, that was designed based on a known structure of the p53 binding protein - 53BP2 and further synthesized by Friedler et al. [271], demonstrated the capability to bind and stabilize the core domain of both wt and mutp53 protein, restore its native state (chaperone mechanism) and the ability to specifically bind DNA. Furthermore, CDB3 was shown to induce expression of p53 target genes (*CDKN1A/p21*, *MDM2*, *GADD45*) and subsequent apoptosis in human osteosarcoma and lung cancer cells expressing mutp53 [272]. Although cell

death was not observed in cancer cells with wtp53 upon treatment with CDB3, the compound sensitized these cells to IR, leading to p53-dependent apoptosis.

Boeckler et al. [260] performed a structure-based *in silico* screening of chemical library and selected a number of compounds to be tested for their ability to bind p53 with Y220C mutation (Y220C-p53), which is one of the most frequently found p53 mutations in cancer. Further biophysical characterization of the selected molecules led to the identification of a carbazole derivative (PhiKan083) as a compound possessing better binding affinity for Y220C-p53, compared to other candidates, and leading to stabilization of the mutant protein. The solved crystal structure of the Y220C-p53-PhiKan083 complex allowed better understanding of the mode of PhiKan083 binding to Y220C-p53 [273, 274], which might be useful for a rational design of new compounds that would selectively bind p53 mutants, but not wtp53, with high affinity. Another small molecule targeting Y220C-p53 mutant, PK7088, identified by Liu et al. [261], was shown to restore wild-type conformation of this p53 mutant, induce expression of p21, Noxa and PUMA and lead to G2/M cell-cycle arrest and apoptosis *in vitro*.

The compound belonging to piperazinyl-quinazolines, SCH529074, bound DNA-binding domain and restored wild-type conformation as well as promoter-specific DNA binding activity of R273H, R175H and R249S p53 mutants (colorectal, endometrial, ovarian cancer and colon adenocarcinoma cell lines), while also stabilizing p53 and preventing its MDM2-mediated ubiquitination and proteasomal degradation [262]. SCH529074 also induced upregulation of p53 targets - p21 and Bax, and led to apoptosis of cancer cell lines with the indicated p53 mutations *in vitro*, whereas oral administration of SCH529074 in a colon adenocarcinoma xenograft model significantly suppressed tumor growth, compared to vehicle control.

Ellipticine is a naturally occurring alkaloid, isolated from *Apocynaceae* plants, and its derivatives are known to be cytotoxic for cancer cells from a variety of solid tumors and haematological malignancies, in particular, induce cell cycle arrest and apoptosis mainly through intercalation into DNA and inhibition of DNA topoisomerase II activity [263]. Recently, however, ellipticine was shown to restore native conformation and transcriptional activity of p53 mutants (R175H, R248W, R249S, R273H, and R281G) [275] and cause upregulation of MDM2 and p21 expression, while its ability to induce apoptosis varied among different p53 mutants. More recently, mutp53 transactivation by ellipticine was demonstrated to sensitize lymphoma cell lines to doxorubicin-induced cell death [276], and also induce increased cytotoxicity, when combined with 5-fluorouracil (5-FU) in colon cancer cells, along with depletion of putative cancer stem cells, population of which is enriched following treatment with 5-FU alone [277].

The agent called WR1065 is the active form of amifostine, a drug used in clinic for cell protection against DNA damage and cytotoxicity induced by ionizing radiation and chemotherapy. Importantly, amifostine selectively protects normal tissue and does not interfere with cytotoxicity in tumor cells [278]. Based on preliminary findings that amifostine is able to partially restore transcriptional activity of several p53 mutants, North et al. tested its active form, WR1065, in a squamous cell carcinoma cell line expressing V272M p53 mutant [279]. They found that WR1065 restored wild-type conformation of p53 mutant in this cell line as well as its DNA-binding activity and also induced expression of a number of p53 targets (p21, GADD45) and cell cycle arrest in G1 phase. Later this group demonstrated that wtp53 activation by WR1065 in breast carcinoma cell line occurs through a JNK-dependent p53 phosphorylation at Thr81, which is associated with p53 accumulation as a result of inhibition of its degradation [280]. The WR1065-induced activation of wtp53 was also shown to be independent of DNA damage and suggested to be

mediated by direct reduction of p53 thiol groups [281], thus promoting its redox-dependent DNA-binding activity. In fact, the oxidation of p53 mutants is considered one of the major biochemical factors leading to their improper folding and preventing them from reverting to wild-type conformation [282]. Therefore, maintaining mutp53 in a reduced state may help to achieve the native p53 folding - a mechanism also reported for other small molecule compounds, discussed below.

The screenings of the datasets of low molecular weight compounds for their ability to selectively suppress proliferation of cancer cells possessing mutp53, but not cells with wtp53, conducted by Bykov et al. resulted in identification of a number of compounds, including PRIMA-1 (p53 reactivation and induction of massive apoptosis), STIMA-1 (SH group-targeting compound that induces massive apoptosis) and MIRA-1 (mutp53-dependent induction of rapid apoptosis). All three compounds are able to participate in reactions of Michael addition (Michael acceptors) and, therefore, can bind and modify cysteine thiols in proteins [256, 283]. MIRA-1 (1-(propoxymethyl)-maleimide) [257] induced mutp53-dependent growth inhibition and reduced colony forming ability in ovarian carcinoma or lung adenocarcinoma cell lines expressing R175H or R273H p53 mutant to a significantly larger extent, compared to p53 *null* cells. Restoration of native conformation of p53 mutants by MIRA-1 led to transcriptional upregulation of p53 targets (MDM2, p21), subsequently leading to activation of caspases and cell death through apoptosis. However, variation in the ability of MIRA-1 to restore the specific DNA-binding activity of different p53 mutants as well as toxicity of MIRA-1 analog observed in human tumor xenografts *in vivo*, suggests the need for optimization of properties of this compound and further characterization of its mechanism of mutp53 reactivation. STIMA-1 compound (2-vinylquinazolin-4-(3H)-one) [256] inhibited growth of lung carcinoma and osteosarcoma cells

expressing mutp53 (R175H or R273H), and the effect was significantly more profound, in comparison with p53-*null* or wtp53 expressing cancer cells or normal human fibroblasts. Similar to MIRA-1, STIMA-1 induced transactivation of p53 targets (p21, PUMA, Bax) and caspases, causing apoptosis in a mutp53-dependent manner. However, due to the observed toxicities *in vivo* and non-favorable physical properties, such as poor solubility [283], further development of both MIRA-1 and STIMA-1 molecules was terminated.

1.3.5 PRIMA-1 and PRIMA-1^{MET} (APR-246)

The PRIMA-1 (p53 reactivation and induction of massive apoptosis) compound, and its methylated analog PRIMA-1^{MET} (APR-246), were identified by Bykov et al. [284, 285] as a result of a screening of the NCI library of low-molecular-weight compounds in a cell-based assay (Figure 1.9), showing selective inhibition of growth and induction of apoptosis in cancer cells carrying mutp53 by restoring wild-type conformation of mutp53 leading to its transcriptional transactivation, reactivation of sequence-specific DNA binding and induction of expression of several p53 targets (such as p21, MDM2). PRIMA-1 and PRIMA-1^{MET} also inhibited growth of tumor xenografts in a mutp53−dependent manner.

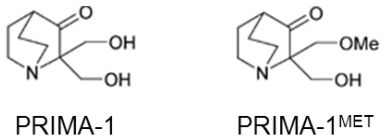

Figure 1.9. Structural formulas of PRIMA-1 and PRIMA-1^{MET} (adapted from [285])

Importantly, PRIMA-1^{MET} is the first compound of its class that reached clinical stage, and was shown to be safe and well-tolerated by patients with hematologic malignancies and prostate cancer in phase I study (maximum tolerated dose = 60 mg/kg), while also demonstrating favorable

pharmacokinetic profile and p53-dependent biological effects (cell cycle arrest, increased apoptosis, upregulation of p53 target genes in tumor cells) [286]. Furthermore, PRIMA-1^{MET} combined with platinum compounds was reported to have synergistic effect in ovarian cancer cell lines as well as tumor xenografts [287] and is currently tested in phase II study for patients with recurrent platinum sensitive p53 mutant high-grade serous ovarian cancer [288].

Molecular mechanisms. Mechanistically, both PRIMA-1 and PRIMA-1^{MET} are decomposed to methylene quinuclidinone (MQ) after 4 to 24 hours *in vitro* and at 1 hour after intravenous injection in mice (Figure 1.10) [289]. MQ is a Michael acceptor capable of reacting covalently with thiols in p53 protein, thus, stabilizing it in the wild-type conformation, the modification shown to occur more readily in recombinant and cellular unfolded mutp53 than in correctly folded wild-type protein. In addition, PRIMA-1 was able to bind unfolded wtp53 proportionally to the degree of its unfolding.

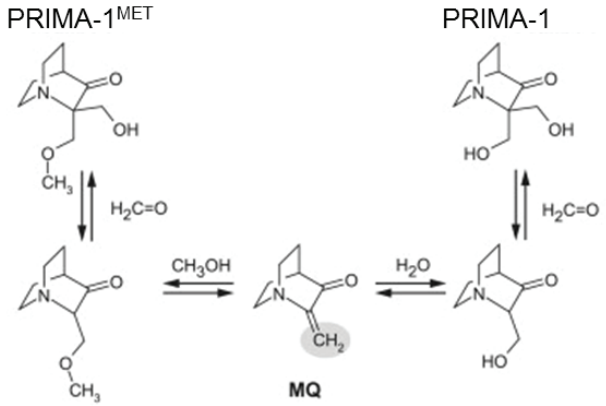

Figure 1.10. Conversion of PRIMA-1 and PRIMA-1^{MET}. A reactive chemical group forming a classical Michael acceptor is indicated (grey circle) in methylene quinuclidinone (MQ) (adapted from [289]).

Mutant p53-associated effects. Since PRIMA-1 and PRIMA-1^{MET} identification, multiple studies have demonstrated the selective cytotoxicity of both compounds in mutp53 carrying tumor cells derived from various cancer types, including lung [290], breast [291], colorectal [292], cervical [293], bladder [294], pancreatic [295] cancer, osteosarcoma [284], head and neck squamous cell carcinoma [296], thyroid carcinoma [297], etc. [298]. PRIMA-1 or PRIMA-1^{MET}-induced restoration of wild-type conformation to mutp53 typically causes upregulation of p53 targets and pro-apoptotic genes, such as Noxa, PUMA, p21, Bax, GADD45, leading to cell cycle arrest and activation of mitochondrial apoptosis pathway associated with activation of caspases, PARP cleavage and release of mitochondrial cytochrome *c* resulting in apoptosis [289, 290, 292, 294, 296, 299-303]. Indeed, the list of genes differentially expressed in mutp53 osteosarcoma cells following treatment with PRIMA-1^{MET}, as shown by microarray analysis, included the ones involved in the cell cycle and proliferation (at an early time point) as well as the cell death pathway, endoplasmic reticulum (ER) stress and rearrangement of the cytoskeleton (at a later time point) [304]. Interestingly, Aryee et al. [305] reported various sensitivities of three Ewing sarcoma cell lines established from the same patient at different stages of the disease, while the comparative analysis of their transcriptome following treatment revealed differential expression of a number of p53 targets and apoptosis-associated genes (such as *APOL6*, *PENK*, *PCDH7*, *MST4*) in PRIMA-1^{MET}-sensitive cell line in comparison with more resistant ones, indicating that PRIMA-1^{MET} effects may be cellular context dependent.

In addition, a mutp53-dependent increase in expression of microRNA-34a, belonging to a microRNA-34 family, whose members are involved in regulation of cell proliferation and apoptosis, was shown to be necessary for PRIMA-1-induced apoptosis in p53 mutant lung cancer cell lines [306]. The effects of PRIMA-1/ PRIMA-1^{MET} treatment on the expression of vascular

endothelial growth factor (VEGF) by tumor cells were reported in several works. In particular, PRIMA-1 was shown to block the hormone-induced expression of VEGF in mutp53 expressing breast cancer cell lines and human tumor xenografts in nude mice [300, 307, 308]. The previous reports that wtp53 is able to suppress VEGF expression let the authors argue that VEGF inhibition in breast cancer cells is an indicator of PRIMA-1-induced restoration of wild-type functions to mutp53. In contrast, Lambert et al. [304] found a strong upregulation of VEGF mRNA following treatment of osteosarcoma cell line expressing exogenous mutp53 with PRIMA-1^{MET}, which was suggested to occur in a mutp53-independent manner as a result of the ER stress induced by the drug. All these findings suggest the complex nature of mechanisms, by which PRIMA-1 and PRIMA-1^{MET} can induce cytotoxicity and cell death in tumor cells expressing mutp53.

Effect on GOF mutp53. Several studies analyzed the effects of PRIMA-1 and PRIMA-1^{MET} tumor cells expressing mutp53 with gain-of-function activity. In particular, Shi et al. [309] demonstrated that silencing of R249S mutp53 expression (by siRNA) in hepatocellular carcinoma cells enhanced the cytotoxicity of PRIMA-1, suggesting that siRNA suppressed the protective and pro-survival GOF activity of this mutp53, while PRIMA-1 exerted its cytotoxic effects through p53-independent mechanisms. Similarly, Russo et al. reported increased cytotoxicity of PRIMA-1 in human breast carcinoma cells possessing GOF R280K mutp53 following transient transfection with p53 siRNA. Importantly, a higher dose of PRIMA-1 induced a decrease in mutp53 levels in the cells transfected with control siRNA and completely diminished residual mutp53 expression in p53 siRNA-transfected cells, potentially through triggering degradation of mutp53 [310]. A shift towards cells with lower expression of mutp53 was observed by flow cytometry following PRIMA-1^{MET} treatment of lung adenocarcinoma cells expressing exogenous R175H mutp53 compared to heterogeneous cell population with different levels of mutp53 in the untreated control

[285]. Based on these findings, the authors suggest that tumor cells with high levels of mutp53 are more sensitive to PRIMA-1^{MET} and are, therefore, selectively eliminated as a result of the treatment.

Alternative mechanisms of action. In contrast to initial reports of mutp53-dependent manner of action, numerous studies observed PRIMA-1/ PRIMA-1^{MET}-induced apoptosis in tumor cells irrespective of their *TP53* status. Particularly, it was suggested that MQ is able to bind cysteines not only in p53 protein, but also in glutathione (GSH), an important cellular antioxidant (Figure 1.11). The capability of MQ to bind GSH and possibly also free intracellular cysteines required for GSH synthesis, led to the decrease in GSH content in the myeloma cells (mutp53, wtp53 or p53-*null*) and a shift in the ratio of its reduced (GSH) to oxidized (GSSG) forms, which changed cellular redox potential and induced reactive oxygen species (ROS) production, inhibiting GSH production even further and resulting in apoptosis [311]. Similar results were obtained by the group of Mohell et al. using ovarian cancer cells, in which both PRIMA-1^{MET} and MQ reduced glutathione levels, while the ability of MQ to physically bind glutathione was also demonstrated [287]. Thioredoxin reductase 1 (TrxR1), an enzyme also involved in regulation of cellular redox balance, was shown by Peng et al. as another example of p53-independent target of PRIMA-1^{MET} [312]. In this study treatment of lung adenocarcinoma and osteosarcoma cells carrying exogenous mutp53 or parental p53-*null* cell lines with PRIMA-1^{MET} inhibited cellular TrxR1 activity irrespective of *TP53* status. The authors, therefore, suggested that TrxR1 inhibition might not only explain to some extent the apoptosis induced by this compound in mutp53-lacking cells, but also contribute to its cytotoxicity in mutp53 cells. Grellety et al., in turn, confirmed the ability of PRIMA-1^{MET} to induce ROS, subsequently causing a loss of mitochondrial membrane potential and cell death, in soft-tissue sarcoma cell lines with different status of p53 [313]. However, having

focused on the downstream effects of ROS induction rather than the factors leading to it, they showed an increased JNK phosphorylation in both mutp53 and wtp53 cell lines, which was suggested to occur in a ROS-dependent manner and serve as an important mechanism of cell death. The role of JNK activation was also suggested by Li et al., who reported a significant decrease in PRIMA-1-induced apoptosis in the presence of JNK inhibitor in colorectal cancer, although only mutp53 cell lines were tested [292]. In contrast, Wang et al. showed a PRIMA-1-induced inhibition of p53 binding to the promoter of upstream activator of JNK signaling pathway (*MAP4K4*) in breast cell lines independent of their p53 status and, therefore, concluded that apoptosis caused by this compound occurs through upregulation of pro-apoptotic proteins PUMA and Bax, rather than JNK activation in breast cancer [291].

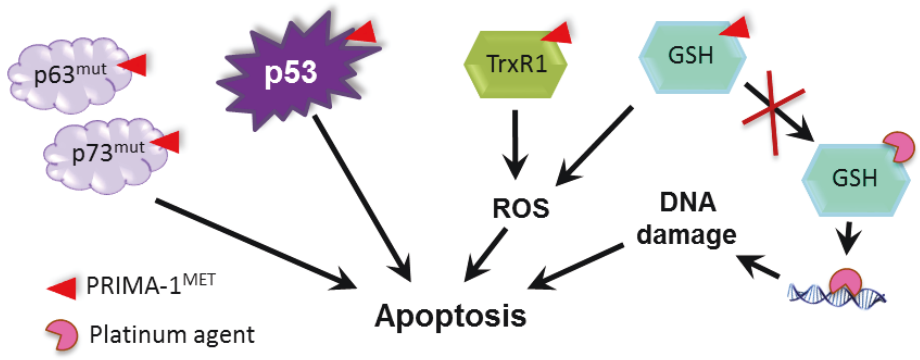

Figure 1.11. PRIMA-1^{MET} mechanisms of action.

Additional p53-independent mechanism of action of PRIMA-1^{MET} was described by Rokaeus et al. The authors showed that PRIMA-1^{MET} targeted the mutant forms of p63 and p73, belonging to p53 family of proteins, and rescued their pro-apoptotic functions, including DNA-binding activity and ability to activate expression of target genes in transfected p53-*null* lung adenocarcinoma and osteosarcoma cell lines [314]. Similarly, Saha et al. [315] suggested that PRIMA-1^{MET}-induced cell death in multiple myeloma cells carrying wild-type, mutant, or null p53

occurred in a p73-dependent manner, since p73 expression was upregulated after the treatment, while its silencing by siRNA attenuated apoptosis in these cell lines, whereas p53 knockdown had little effect. Interestingly, an increase in the levels of Noxa detected following the treatment and shown to be required for apoptosis, was diminished in cells, in which p73 was silenced, indicating that p73 is an upstream regulator of Noxa expression.

Several studies showed that PRIMA-1^{MET} and PRIMA-1^{MET} are able to induce heat shock protein response characterized by upregulation of some members of heat-shock family of proteins (HSP), whose normal functions include regulation of normal protein folding, prevention of improper associations and aggregation [316]. In particular, PRIMA-1^{MET} treatment resulted in increased levels of Hsp70 chaperone protein in lung adenocarcinoma cells with exogenous mutp53 or p53-*null*, and in colon carcinoma cell lines carrying endogenous mutp53. Interestingly, Hsp70 and mutp53 translocation to nucleoli was observed in mutp53, but not in null p53 cell lines, raising the possibility of Hsp70 involvement in PRIMA-1^{MET}-induced mutp53 refolding [317]. Hsp70 upregulation following PRIMA-1^{MET} treatment was also found in multiple myeloma cells irrespective of their *TP53* status [315]. A study by Rehman et al. [299] showed PRIMA-1-induced translocation of mutp53 and another chaperone, Hsp90 (α isoform), to the nucleus of mutp53 breast cancer cell lines, accompanied by physical interaction between these two proteins as revealed by co-immunoprecipitation assay. The authors suggested that Hsp90α might be important for restoration of the transcriptional transactivation function of mutp53.

Although other studies did not identify the mechanisms, they showed that PRIMA-1^{MET} induced apoptosis in both wt and mutp53 expressing cells in a p53-dependent manner - Ewing sarcoma [305] and colorectal cancer [318]. The role of $p14^{ARF}$, an inhibitor of MDM2-mediated degradation of p53, in the response to PRIMA-1 was suggested by Paul et al. who showed that

leukemic cells, obtained from patients with normal karyotype *de novo* acute myeloid leukemia, with low $p14^{ARF}$ mRNA levels and wtp53 were more sensitive to this compound compared to cells with high $p14^{ARF}$ expression [319]. Additionally, Shchors et al. showed that PRIMA-1 induced inhibition of proliferation and activation of p53 targets occurred only in mutp53 GBM cell lines possessing wild-type $p14^{ARF}$, but not in $p14^{ARF}$ *null* cells [320]. Another study used p53-*null* glioma cells transfected with different p53 mutants (R175H, R248W and R273H) and showed that PRIMA-1 induced cell death irrespective of the mutation type and independent of p53 expression [258]. The previously mentioned or alternative p53-independent effects of PRIMA-1 and PRIMA-1^{MET} might explain the cytotoxicity of these compounds in p53-*null* cells.

Finally, an alternative type of cell death - autophagy, rather than apoptosis, was observed in breast and colon cancer cell lines treated with PRIMA-1 irrespective of their p53 status [321], and in mutp53 expressing soft-tissue sarcoma cell lines after PRIMA-1^{MET} treatment, in the latter case potentially as a result of the oxidative stress induced by this compound [313].

Synergy with therapeutic agents. The synergistic effects of both PRIMA-1 and PRIMA-1^{MET} when combined with other therapeutic agents were shown in multiple studies. In particular, mutp53-dependent synergy of PRIMA-1 with cisplatin (platinum agent) and doxorubicin (anthracycline antibiotic) was detected in osteosarcoma and non-small cell lung cancer (NSCLC) cells, leading to increased activation of caspases, induction of Bax and PUMA, suppression of colony formation *in vitro* and tumor growth delay *in vivo* [285]. PRIMA-1^{MET} also showed strong synergy with cisplatin in inducing apoptosis and ROS in ovarian cancer cell lines *in vitro* and an additive effect in tumor xenografts, and was able to re-sensitize drug-resistant ovarian cancer cells to doxorubicin and gemcitabine (nucleoside analog) [287]. The authors suggest that there is a dual mechanism of synergy, including both p53-dependent and independent effects. Of note, p53-

dependent effects occur through MQ binding to cysteines of p53 protein resulting in refolding of its core domain and restoration of p53 wild-type functions required for induction of apoptosis in response to treatment with platinum compounds. The latter effects, in turn, might be due to the ability of MQ to decrease glutathione concentration in the cells by directly inhibiting its synthesis or by inducing ROS production, which allows for a greater amount of platinum drugs to bind DNA, rather than glutathione, and increases their cytotoxicity (Figure 1.11). PRIMA-1^{MET} synergized with doxorubicin in multiple myeloma cell lines *in vitro*, and with dexamethasone (corticosteroid) both *in vitro* and *in vivo* (tumor xenografts) [315]. Interestingly, synergistic effect of PRIMA-1 with other p53-reactivating molecules, specifically the inhibitors of the p53-MDM2 interaction, such as RITA and Nutlin-3 was reported for acute myeloid leukemia [322] and pancreatic cancer cell lines [295], respectively. Importantly, PRIMA-1^{MET} was able to radiosensitize prostate cancer cells as reflected by an increased inhibition of colony formation, when used in combination with ionizing radiation [323], providing a strong rationale for testing this compound in GBM, for which RT is a key component of standard treatment.

1.4 HYPOTHESIS AND RESEARCH OBJECTIVES

While MGMT expression and *TP53* alterations are key determinants of GBM chemoradioresistance, understanding the potential effect of MGMT expression on p53 and their interplay specifically in the context of expression of mutp53 is still lacking. Likewise, the efficacy of PRIMA-1^{MET}, its mechanism of action and its potency as a radiosensitizer in GBM have not been investigated. In view of the aforementioned considerations we concentrated our efforts around the following hypothesis: there is a potential interplay between MGMT and p53, which may affect the response of GBM cells to PRIMA-1^{MET} alone and in combination with IR.

More specifically, as outlined below, three major objectives of this thesis were:

Objective 1: To investigate the relationship between MGMT and p53 using data from publicly available cell lines datasets and isogenic GBM cell lines for expression of MGMT

Objective 2: To test the efficacy of PRIMA-1^{MET} and determine its molecular mechanisms of action in GBM while taking into account *TP53* status and MGMT expression levels

Objective 3: To assess PRIMA-1^{MET} potency as a radiosensitizer in GBM cells with different MGMT levels and p53 status.

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Chapter 2.

SENSITIVITY TO PRIMA-1^{MET} IS ASSOCIATED WITH DECREASED MGMT IN HUMAN GLIOBLASTOMA CELLS AND GLIOBLASTOMA STEM CELLS IRRESPECTIVE OF P53 STATUS

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DOI: 10.18632/oncotarget.11197

2.1 ABSTRACT

Alterations of the *TP53* tumor suppressor gene occur in ~30% of primary GBM with a high frequency of missense mutations associated with the acquisition of oncogenic "gain-of-function" (GOF) mutant (mut)p53 activities. PRIMA-1^{MET}/APR-246, emerged as a promising compound to rescue wild-type (wt)p53 function in different cancer types. Previous studies suggested the role of wtp53 in the negative regulation of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT), a major determinant in resistance to therapy in GBM treatment. The potential role of MGMT in expression of p53 and the efficacy of PRIMA-1^{MET} with respect to *TP53* status and expression of MGMT in GBM remain unknown. We investigated response to PRIMA-1^{MET} of wtp53/MGMT-negative (U87MG, A172), mutp53/MGMT-positive U138, LN-18, T98/Empty vector (T98/EV) and its isogenic MGMT/shRNA gene knockdown counterpart (T98/shRNA). We show that MGMT silencing decreased expression of mutp53/GOF in T98/shRNA. PRIMA-1^{MET} further cleared T98/shRNA cells of mutp53, decreased proliferation and clonogenic potential, abrogated the G_2 checkpoint control, increased susceptibility to apoptotic cell death, expression of GADD45A and sustained expression of phosphorylated Erk1/2. PRIMA-1^{MET} increased expression of p21 protein in U87MG and A172 and promoted senescence in U87MG cell line. Importantly, PRIMA-1^{MET} decreased relative cell numbers, disrupted the structure of neurospheres of patient-derived GBM stem cells (GSCs) and enabled activation of wtp53 with decreased expression of MGMT in MGMT-positive GSCs or decreased expression of mutp53. Our findings highlight the cell-context dependent effects of PRIMA-1^{MET} irrespective of p53 status and suggest the role of MGMT as a potential molecular target of PRIMA-1^{MET} in MGMT-positive GSCs.

2.2 INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and deadliest malignant primary brain tumor in adults [1-3]. Despite aggressive treatment involving surgery, radiation therapy (RT) and the alkylating agent temozolomide (TMZ), the prognosis for patients diagnosed with GBM remains extremely poor with a median survival of 14.6 months and only 10% of patients alive at 5 years after adjuvant chemoradiation [4-7]. The DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) removes methyl adducts from the O(6) position of guanine and, therefore, interferes with cytotoxicity of alkylating agents, including TMZ [8]. Over the last two decades, several groups identified the role of brain tumor initiating (stem) cells (BTICs) or glioma/ GBM stem cells (GSCs), as a highly tumorigenic subpopulation of cancer cells able to self-renew and generate a differentiated progeny [9, 10]. GSCs promote therapeutic resistance and drive tumor recurrence further challenging response to standard therapy [11]. In addition to the biological complexity of GBM, landmark genomic and transcriptomic studies revealed that GBM encompasses clinically relevant molecularly heterogeneous diseases classified into “proneural”, “neural”, “classical”, and “mesenchymal” subtypes [12].

The p53 tumor suppressor protein regulates cell cycle progression, DNA repair, apoptosis and senescence in response to various stress stimuli through transcriptional activation of multiple target genes, including $p21^{Waf1/Cip1}$, the growth arrest and DNA damage 45 (GADD45A), Bax, Noxa, PUMA, KILLER/DR5, Fas etc. [13, 14]. Alterations in *TP53* gene are reported in about 25-30% of primary GBM [15] with increased onset of *TP53* mutations in the “proneural” subtype [12, 16]. The majority of *TP53* mutations in human cancer are missense mutations that commonly occur within the DNA-binding domain of p53 resulting in disruption of p53 DNA-binding activity and impaired ability to regulate target genes and transactivate the p53 antagonist MDM2. Inhibition of

MDM2-mediated mutant (mut)p53 degradation contributes within an intricate complex network to stabilization and increased expression of mutp53 protein [17, 18]. *TP53* mutations lead to abrogation of the wild-type (wt) activity of p53 and its function as a tumor suppressor gene or act as dominant negative (DN) inhibitors able to form cotetramers with co-expressed wtp53. Remarkably, *TP53* missense mutations may confer novel oncogenic properties described as mutp53 "gain-of-function" (GOF), which encompass p53 activities in the absence of co-expressed wtp53 and lead to more aggressive behavior of tumor cells such as promoting invasion, preventing apoptosis and increasing resistance to anticancer treatments [19-21]. Intriguingly, previous studies suggested the role of wtp53 in the negative regulation of MGMT levels in different human cancer cell lines including GBM [22, 23]. As a corollary, the strategy to rescue wtp53 function may concomitantly lead to decreased levels of MGMT in GBM tumors, thereby eluding resistance to alkylating agents currently used as a standard therapy in GBM treatment.

Small molecules designed to rescue wtp53 function have emerged as a potentially promising strategy to circumvent the proliferative and anti-apoptotic advantages gained through loss of p53 tumor suppressor function in different types of cancer [24-26], including gliomas [27, 28]. PRIMA-1 (p53 reactivation and induction of massive apoptosis) and its methylated and more active form PRIMA-1^{MET} (APR-246) identified by Bykov and colleagues restore mutp53 activity by promoting proper folding of the mutant protein [29, 30]. PRIMA-1^{MET} and PRIMA-1 were also shown to selectively inhibit growth and induce apoptosis in ovarian, osteosarcoma and lung cancer cell lines, harboring mutp53 *in vitro* and *in vivo* [29, 31, 32]. However, PRIMA-1^{MET} demonstrated cytotoxicity and cellular context dependency regardless of *TP53* mutational status of tumor cells in several cancer types (prostate, melanoma) [33, 34]. From a clinical point of view, PRIMA-1^{MET} is the only mutp53 reactivation compound, which showed safety, favorable pharmacokinetic

profile and p53-dependent biological activity in phase I study in patients with hematologic malignancies and prostate cancer [35]. Recently, its combination with platinum-based therapy in phase Ib/II proof of concept study provided supporting evidence for the continuation of the phase II study for patients with recurrent p53 mutant high-grade serous ovarian cancer [36].

While alterations of *MGMT* and *TP53* are key determinants of GBM chemoradioresistance, understanding the potential effect of MGMT expression on p53 specifically in the context of expression of mutp53 is still lacking. Likewise, the efficacy of PRIMA-1^{MET} and its mechanism of action in GBM have not been investigated while taking into account both *TP53* status and MGMT expression levels. In this study, we investigated the potential causal relationship between MGMT and mutp53, and how MGMT may affect mutp53 GOF activities in response to PRIMA-1^{MET}. To this end, we used GOF mut*TP53* [20] isogenic cell lines with at least 90% knockdown of MGMT in addition to other established GBM cell lines with different p53 status and MGMT levels. We assessed whether MGMT affects the cytotoxicity of PRIMA-1^{MET}, its antiproliferative activity, its effect on clonogenic potential and the cell cycle. We also analyzed the molecular pathways underlying its cellular effects.

Given the potential role of GSCs in resistance to treatment and tumor relapse, we further investigated the effect of PRIMA-1^{MET} on patient-derived GSCs with different p53 status and MGMT levels. Our findings highlight the cell-context dependent effects of PRIMA-1^{MET} irrespective of p53 status in established GBM cell lines and GSCs. Despite their inherent genetic cell heterogeneity, we provide the first evidence that the cytotoxicity of PRIMA-1^{MET} is associated with activation of wtp53 and decreased expression of MGMT in MGMT-positive GSCs, while expression of mutp53 protein was decreased in MGMT-negative GSC line.

2.3 MATERIALS AND METHODS

2.3.1 Expression and mutation analysis of CCLE and NCI-60 cell lines.

Normalized mRNA expression data (z-score values) for CCLE human cancer cell lines were extracted from the CCLE portal (available at http://www.broadinstitute.org/ccle) [37]. Data (log2 values) from reverse-phase protein lysate microarrays (RPLA) for NCI-60 panel of human cancer cell lines were extracted from CellMiner database (version 1.61) [38]. The information on *TP53* mutations in analyzed cell lines was obtained from the p53 website [39, 40], COSMIC [41, 42], and literature [43, 44]. SNB-19 glioma (derived from the same individual as U251 cell line [42]), SK-OV-3 ovarian (p53 mRNA and protein are undetectable [40]), OVCAR-5 ovarian (controversial p53 status), NCI-ADR-RES ovarian (similar to OVCAR-8 cell line), HL-60 leukemia (p53 null) [45], MDA-MB-435 and MDA-N melanoma (similar to M14 melanoma cell line [46]) cancer cell lines were excluded from the analyses of the NCI-60 and CCLE (SNB-19, SK-OV-3, MDA-MB-435, HL-60) datasets.

2.3.2 Cell culture and drug treatment

The U87MG, T98G, A172, U138 and LN-18 GBM cell lines were obtained from American Type Culture Collection. T98G-based model described in [47] was used, where cells were transfected with plasmid vector encoding shRNA against MGMT (T98/shRNA) or with empty vector (T98/EV). The laboratory of Dr. Thierry Muanza (McGill University) kindly provided U87MG cells stably transfected with a plasmid carrying exogenous MGMT (U87/MGMT) or an empty vector (U87/EV) (transfection by Dr. Jad Ashami at the laboratory of Dr. Rolando Del Maestro). Established GBM cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; standard medium). GBM specimens

used in this study were obtained from patients undergoing surgical treatment at the Montreal Neurological Hospital, in accordance with Institutional Review Board (IRB)-approved protocols. The diagnosis of GBM was made by a neuropathologist. GSCs isolated from cancer specimens were established and grown in neurosphere cultures as previously described [48]. GSCs expanded in neurosphere cultures retained self-renewal capacity in serum-free media, expressed neural stem cell markers, such as CD133 and nestin, and had the ability to differentiate in serum-containing growth media. 48EF GSCs were kindly provided by Dr. Samuel Weiss (University of Calgary). GSCs were maintained in neural stem cell complete medium NeuroCult NS-A Basal Medium with NeuroCult NS-A proliferation supplement (STEMCELL Technologies Inc., BC, Canada), Heparin (STEMCELL Technologies, BC, Canada), Epidermal Growth factor (EGF, 20 ng/ml) and Fibroblast Growth factor 2 (FGF-2, 20 ng/ml) (Life Technologies Inc., ON, Canada). All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2. Cells were treated with PRIMA-1^{MET} (Tocris Bioscience, Bristol, UK) dissolved in DMSO at varying doses in standard medium for 24 hours and then left in drug-free medium for additional time depending on the assay used. Cells treated with DMSO were used as a control.

2.3.3 RNA isolation, PCR and sequencing

Total RNA was isolated from GBM cells using TRIzol® reagent (Thermo Fisher Scientific Inc., Waltham, MA USA) according to the manufacturer's directions. The RNA was dissolved in 30 µl of DNase/RNase-free distilled water (Thermo Fisher Scientific Inc.). Reverse transcription was performed with 0.5 µg of total RNA using QuantiTect Reverse Transcription Kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's directions. The regions corresponding to exons 3-4 (467 bp), exons 5-7 (498 bp) and exons 7-11 (532 bp) were amplified using the primers specific for sequences flanking each region. Amplification was performed in a 50 µl of a

mixture containing AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific Inc.), 5 µl of cDNA and 0.5 µM of each primer. The amplification was carried out in a 2720 Thermal Cycler (Applied Biosystems) with an initial denaturation at 95^0C for 5 min and followed by 35 cycles at 94^0C for 15 s, 55^0C for 1 min, 72^0C for 1 min and a final extension for 10 min at 72^0C. Amplicons were sequenced at the McGill University and Genome Quebec Innovation Centre using the same pairs of primers on an Applied Biosystems 3730xl DNA Analyzer (Sanger DNA sequencing).

***TP53* gene exon**	**Forward (For) and reverse (Rev) primer sequences (5′→3′)**
Exons 3-4	For: CAGTCAGATCCTAGCGTCG Rev: CGGTAGATGTTCGTCAGT
Exons 5-7	For: CAGAAAACCTACCAGGGC Rev: CCTGCCTTGTCGAAACTC
Exons 7-11	For: GACATAGTGTGGTGGTG Rev: GAGGTGAAGAACAAGGGG

2.3.4 Trypan blue exclusion cell viability assay

GBM cell cultures were subjected to varying doses of PRIMA-1^{MET} for 24 hours (24-hour time point) and then incubated for additional 24 (48-hour time point) or 48 hours (72-hour time point) in a drug-free medium. After that cells were washed with phosphate-buffered saline (PBS), trypsinized for 5 min and then neutralized by the addition of new complete medium. PBS used for washing was also collected to avoid losing easily detaching apoptotic cells (established GBM cell lines). Cells were pelleted by centrifugation at 1500 g for 10 min. The supernatant was aspirated and the cells were resuspended in a suitable volume of growth media (50-500 µl). The cell number and a ratio of dead cells with disrupted membranes (blue cells) to total number of cells was counted in triplicate for each well of plated cells using automated cell counter TC-10 (Bio-Rad Laboratories, Inc., Mississauga, ON, Canada) or automated Vi-CELL Cell Viability Analyzer

(Beckman Coulter, Inc., Mississauga, ON, Canada). Cell number is represented as a percentage relative to cell number in control (100%). Percentage of viable (live) cells is represented in relation to the total cell number in each experimental condition.

2.3.5 MTT assay

Cells were plated in 96-well plates at a density of 2500 cells per well in standard DMEM medium and allowed to adhere overnight at 37°C in 5% CO_2. After that the cells were treated with PRIMA-1^{MET} at varying concentrations for 24 hours and left in drug-free medium for additional 24 hours before adding MTT. Cell proliferation was measured using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific Inc.). 10 µl of 0.5% MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to each well in the 96-well plates and 100 µl of 10% sodium dodecyl sulfate (SDS) was added 4 hours after adding MTT. After an overnight incubation, the absorbance was read at 570 nm.

2.3.6 Clonogenic assay

Cells were plated in 6-well plates, allowed to adhere overnight and treated with PRIMA-1^{MET} at varying concentrations in standard medium for 24 hours. Then the medium was replaced with drug-free medium and the cells were incubated for additional 7-14 days or until colonies (more than 50 cells) were formed. Cells were then fixed with 10% formalin and stained using 1.5% methylene blue. Colonies of at least 50 cells were counted. The surviving fraction was normalized to the plating efficiency of the corresponding DMSO controls.

2.3.7 Senescence assay

Cells were stained for senescence-associated beta-galactosidase activity (SA-β-Gal) as described by Dimri et al. [49] using Senescence β-Galactosidase Staining Kit (Cell signaling, Danvers, MA, USA) following the manufacturer's protocol. Briefly, cells were seeded in 6-well plate, allowed to adhere overnight, treated with PRIMA-1^{MET} at varying concentrations in standard medium for 24 hours, and left in drug-free medium for additional 120 hours (6 days after the start of treatment). Cells were then washed twice with PBS, fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS, and washed twice in PBS. Cells were stained for overnight in X-gal staining solution (1 mg/ml X-gal, 40 mmol/l citric acid/sodium phosphate (pH 6.0), 5 mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, 150 mmol/l NaCl, 2 mmol/l $MgCl_2$). Light microscopy was used to identify senescent (blue stained) cells. The percentage of SA-β-Gal positive cells was quantified by analyzing at least 400 cells in each experimental condition.

2.3.8 Western blot analysis

Cells were washed twice (established cell lines) or collected (GSCs) with 1X cold PBS and lysed with 1X RIPA buffer (Boston BioProducts, Inc., Ashland, MA, USA) supplemented with 0.2 mM sodium orthovanadate, protease (Sigma-Aldrich, Oakville, ON, Canada) and phosphatase (Roche Diagnostics, QC, Canada) inhibitors cocktails. Proteins (30 µg, Pierce BCA protein assay kit, Thermo Fisher Scientific Inc.) were electrophoretically separated in 12% SDS-PAGE under reducing conditions and transferred onto PVDF membranes. Membranes were probed for MGMT (Santa Cruz, Dallas, TX, USA), $p21^{Waf/Cip1}$ (Cell signaling, Beverly, MA, USA), mutant and wild-type p53 (DO-1, Santa Cruz), β-actin (Sigma-Aldrich, Oakville, ON, Canada), GADD45A (Abcam, Toronto, ON, Canada), cleaved PARP (D64E10, Cell signaling, Beverly, MA, USA),

phosphorylated Erk1/2 (Cell signaling, Beverly, MA, USA), Erk1/2 (Cell signaling, Beverly, MA, USA) according to the manufacturer's recommendations. HRP activity was assayed by chemiluminescence using Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences, Mississauga, ON, Canada). Quantitation of Western blot data was performed using ImageJ software analysis. All data were normalized to loading controls.

2.3.9 Flow cytometry

Cells were treated with PRIMA-1^{MET} for 24 hours, collected, fixed in 70% ethanol, centrifuged, washed twice with PBS, and resuspended in 1 mg/ml RNase A (Sigma-Aldrich), incubated at 37^{0}C for 30 minutes and suspended in 10 µg/ml propidium iodide working solution (Sigma-Aldrich) for 20 minutes at room temperature. Data were acquired on a BD FACSCanto II flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and analyzed with FlowJo (Version 9.6.2, FlowJo, LLC, Ashland, OR, USA) and ModFit LT (Verity Software House, Topsham, ME, USA) software.

2.3.10 Immunofluorescence and confocal microscopy

For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with 100% methanol at -20 °C for 10 min. After blocking with 5% normal serum/ 0.3% Triton™ X-100 in PBS for 60 min at room temperature, cells were incubated with antibody against phospho-p44/42 MAPK (pErk1/2) (Thr202/Tyr204) (Cell signaling) at a working concentration of 1.44 µg/mL, diluted in 1% normal serum/ 0.3% Triton™ X-100 in PBS at 4 °C overnight, and then incubated with fluorescence-conjugated secondary antibody Alexa Fluor 488 (Life technologies) at a working concentration of 8 µg/mL diluted in antibody dilution buffer for 60 min at room temperature in the dark. Nuclei were stained

with 0.1 µg/mL DAPI (Sigma). Images were captured (original magnification 400x) using a Zeiss LSM 780 laser scanning microscope (Carl Zeiss MicroImaging, Göttingen, Germany) and analyzed using ImageJ software (>40 cells analyzed in each experimental condition).

2.3.11 Statistical analysis

We used GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) to generate best-fit sigmoidal dose response curves for IC_{50} determination. Data are reported as mean +/- SD and are representative of at least 3 independent experiments unless otherwise stated. Statistics were performed using either an unpaired two-tailed Student's t-test or one-way ANOVA with a post-hoc test as appropriate. Correlations were estimated by Spearman's or Pearson's correlation methods. P values < 0.05 were considered statistically significant.

2.4 RESULTS

2.4.1 *In silico* analysis of the relationship between MGMT and p53 using publicly available cell lines databases

MGMT is known for its role as a DNA repair protein and loss of its expression as a result of promoter methylation has been associated with increased onset of *TP53* G:C to A:T transition mutations [50-52]. Previous studies reported the role of wtp53 in the negative regulation of MGMT levels in different human cancer cell lines [22, 23]. As a first step to investigate the relationship between MGMT and p53, we used publicly available data for their mRNA levels in the Cancer Cell Line Encyclopedia database (CCLE, http://www.broadinstitute.org/ccle) [37] and the NCI-60 cell line panel. To determine p53 status, we used information from p53 website [39, 40], COSMIC [41, 42], and literature [43, 44]. We excluded several cell lines either for misidentification, p53 null status or conflicting reports for p53 status (described in Materials and methods). There was

no significant correlation between mRNA levels of p53 and MGMT within all the panel of CCLE cancer cell lines originating from 24 primary sites (n = 910), neither for CCLE cancer cell lines harboring all types of alterations of *TP53* (n = 501), or only mutp53 with missense mutations (n = 355). We found a weak but significant positive correlation between mRNA levels (z-score values) of *MGMT* and *TP53* in CCLE panel of human glioma cell lines harboring wt or mutp53 (n = 42, Spearman's rho = 0.36, p value = 0.02) (Appendix Table A1), suggesting a potential specific relationship between MGMT and p53 in primary brain tumors, compared to other types of cancer. There was a significant correlation between mRNA levels of *MGMT* and *TP53* in wtp53 glioma cell lines (n = 17, Spearman's rho = 0.55, p value = 0.024), but not between mRNA levels of *MGMT* and *TP53* in mutp53 glioma cell lines (n = 25). This may reflect the tissue and cellular specificity of mutp53 in addition to the large heterogeneity of mutp53 oncogenic proteins with either DN effect or GOF activities [53].

Expression of mRNA may not reflect protein levels, especially for genes known to be tightly regulated at the post-transcriptional level, such as *TP53* [54] and *MGMT* [55-57]. To investigate the relationship between MGMT and p53 protein expression levels, we used CellMiner database [38], which provides a web interface to access data from reverse-phase protein lysate microarray (RPLA, a platform for quantitative measurement of the amount of particular protein in a large number of biological samples simultaneously [58]) in addition to other gene-based microarray platforms for NCI-60 cell lines across tumors derived from 9 different tissues. We analyzed the highest values for RPLA (log2) provided for p53 isoforms [59] and MGMT (Table 2.1). There was no significant correlation between MGMT and p53 protein levels across all cell lines irrespective of their p53 status (n = 53). Analysis of the mean of RPLA protein levels strictly for cell lines harboring mutp53 revealed a strong and significant negative correlation between MGMT

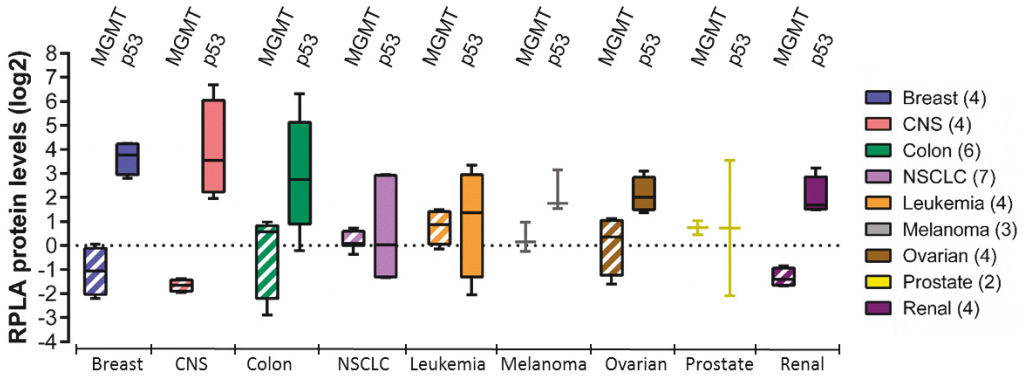
and mutp53 RPLA protein levels across 9 different cancer types (Pearson correlation coefficient = -0.79, p value = 0.012, n = 38). However, we could not analyze with confidence the correlation between mutp53 and MGMT RPLA protein levels within each cancer type including GBM, because of the low number of cancer cell lines with available RPLA information (Figure 2.1).

Table 2.1. MGMT and p53 protein levels (from reverse-phase protein lysate microarrays, RPLA) and *TP53* status in the NCI-60 cell lines panel

Cell line	MGMT	p53	Tissue of origin	TP53 status
BR:BT_549	-1.56	3.38	Breast	MT
BR:HS578T	-0.58	2.8	Breast	MT
BR:MCF7	1.59	0.24	Breast	WT
BR:MDA_MB_231	-2.2	4.18	Breast	MT
BR:T47D	0.05	4.25	Breast	MT
CNS:SF_268	-1.95	6.7	CNS	MT
CNS:SF_295	-1.53	4.08	CNS	MT
CNS:SF_539	-1.53	0.72	CNS	WT
CNS:SNB_75	-1.77	1.96	CNS	MT
CNS:U251	-1.39	3.03	CNS	MT
CO:COLO205	0.78	1.27	Colon	MT
CO:HCC_2998	0.99	-0.2	Colon	MT
CO:HCT_116	-0.52	0.09	Colon	WT
CO:HCT_15	0.58	2.07	Colon	MT
CO:HT29	0.58	4.74	Colon	MT
CO:KM12	-2.88	3.42	Colon	MT
CO:SW_620	-1.98	6.32	Colon	MT
LC:A549	0.37	-0.78	Non-Small Cell Lung	WT
LC:EKVX	0.6	0.04	Non-Small Cell Lung	MT
LC:HOP_62	0.09	-1.3	Non-Small Cell Lung	MT
LC:HOP_92	-0.37	0.85	Non-Small Cell Lung	MT
LC:NCI_H226	0	-1.31	Non-Small Cell Lung	MT
LC:NCI_H23	0.03	2.92	Non-Small Cell Lung	MT
LC:NCI_H322M	0.39	2.96	Non-Small Cell Lung	MT
LC:NCI_H460	-0.65	-0.65	Non-Small Cell Lung	WT
LC:NCI_H522	0.73	-1.33	Non-Small Cell Lung	MT
LE:CCRF_CEM	1.5	3.34	Leukemia	MT
LE:K_562	-0.13	-2.06	Leukemia	MT
LE:MOLT_4	1.16	0.96	Leukemia	MT

LE:RPMI_8226	0.59	1.79	Leukemia	MT
LE:SR	-0.64	0.54	Leukemia	WT
ME:LOXIMVI	-1.99	0.04	Melanoma	WT
ME:M14	0.97	3.16	Melanoma	MT
ME:MALME_3M	-2.21	0.09	Melanoma	WT
ME:SK_MEL_2	-0.24	1.54	Melanoma	MT
ME:SK_MEL_28	0.16	1.75	Melanoma	MT
ME:SK_MEL_5	-2.22	1.23	Melanoma	WT
ME:UACC_257	-0.04	-0.06	Melanoma	WT
ME:UACC_62	-1.49	0.74	Melanoma	WT
OV:IGROV1	-1.6	1.36	Ovarian	MT
OV:OVCAR_3	1.11	3.09	Ovarian	MT
OV:OVCAR_4	-0.15	1.91	Ovarian	MT
OV:OVCAR_8	0.88	2.13	Ovarian	MT
PR:DU_145	1.04	3.55	Prostate	MT
PR:PC_3	0.45	-2.09	Prostate	MT
RE:786_0	-1.68	1.61	Renal	MT
RE:A498	-1.68	0.49	Renal	WT
RE:ACHN	0.44	0.34	Renal	WT
RE:CAKI_1	0.98	-0.07	Renal	WT
RE:RXF_393	-1.6	3.23	Renal	MT
RE:SN12C	-1.19	1.77	Renal	MT
RE:TK_10	-0.85	1.48	Renal	MT
RE:UO_31	1.04	1.27	Renal	WT

Note: WT- wild-type, MT – mutant



Protein	Primary site								
	Breast	CNS	Colon	NSCLC	Leukemia	Melanoma	Ovarian	Prostate	Renal
MGMT	-1.07	-1.65	0.58	0.09	0.875	0.16	0.365	0.745	-1.395
mutp53	3.78	3.555	2.745	0.04	1.375	1.75	2.02	0.73	1.69

Figure 2.1. Correlation between mutp53 and MGMT protein levels in the NCI-60 cell line panel. Box and whisker plots (min-max, the horizontal line indicates median) of MGMT (dashed pattern fill) and p53 (solid fill) protein levels (from reverse-phase protein lysate microarrays, RPLA, log2) in NCI-60 cell lines with mutant *TP53* (n = 38) derived from 9 different cancer types. The median MGMT and p53 protein (RPLA) values for cell lines within each primary site are indicated in the table below the graph. CNS – central nervous system; NSCLC - Non-small cell lung cancer.

2.4.2 MGMT silencing decreased mutp53 protein levels in a GOF mutp53 GBM cell line

To investigate the causal link between MGMT and p53, we analyzed by Western blotting MGMT and p53 protein levels in MGMT knockdown or overexpressing isogenic GBM cell lines. We also used a panel of established GBM cell lines with known p53 status and different MGMT protein levels: MGMT-positive mutp53 GBM cell lines LN-18 (high MGMT protein levels, p53 C238S substitution) and U138 (intermediate MGMT protein levels, p53 R273H substitution) [20, 60] as well as MGMT-negative U87MG and A172 cell lines (Table 2.2).

Table 2.2. *TP53* status and relative p53 and MGMT protein levels in the studied human GBM cell lines

Cell line	*TP53* status	Relative p53 protein level		Relative MGMT protein level	
		Mean±SD	p-value [a]	Mean±SD	p-value [a]
T98/EV	M237I	1.0	-	1.0	-
T98/shRNA	M237I	0.7±0.49	<0.05	0.1±0.34	<0.05
U138	R273H	1.0	n.s.	0.6±0.13	<0.05
LN-18	C238S	0.8±0.54	<0.05	1.2±0.28	<0.05

A172	R72P heterozygous SNP	<0.1	<0.05	0	-
U87MG	Wild-type	<0.1	<0.05	0	-
U87/EV	Wild-type	<0.1	<0.05	0	-
U87/MGMT	Wild-type	<0.1	<0.05	1.6±0.18	<0.05

Note: [a] Protein levels were calculated densitometrically and compared to T98/EV. SNP- single nucleotide polymorphism

We have previously used T98G, a human GBM cell line known to constitutively express high endogenous levels of MGMT and harbor GOF *TP53* mutation [20, 61] and generated stable short-hairpin (sh)RNA-mediated 90% knockdown of endogenous MGMT (T98/shRNA) and its counterpart transfected with empty vector (T98/EV) [47]. As expected, MGMT-knockdown significantly increased sensitivity of T98/shRNA to TMZ treatment in clonogenic survival assay [47]. Sequencing of *TP53* confirmed that both T98/EV and T98/shRNA cell lines possessed p53 mutation in the DNA-binding domain of the protein (M237I substitution) identical to that previously reported in T98G parental cell line (Appendix Table A2) [39, 42]. Because of controversial reports about *TP53* status in A172*,* we used *TP53* sequencing and showed that A172 had R72P heterozygous single nucleotide polymorphism (SNP) in the proline-rich domain of p53, while we confirmed wtp53 status for U87MG (Appendix Table A2) [60].

The p53 protein is maintained at very low levels in cells with wtp53 function, while increased half-life of mutp53 protein enables its detection. Western blotting analysis using the antibody (DO-1) recognizing mutant and wtp53 showed high levels of p53 protein in mutp53 cell lines (T98/EV, T98/shRNA, U138 and LN-18) compared to wtp53 cell lines (A172, U87MG) (Figure 2.2), which showed detectable basal p53 protein levels at longer exposure time (data not shown). Western blotting analysis of p21 confirmed the lack of p21 expression in mutp53 cell lines and its basal

expression in U87MG and A172 cell lines (data not shown). Interestingly, densitometric analysis showed that knockdown of MGMT in T98/shRNA cell line (>90%) was associated with a significant decrease of mutp53 protein levels by 35±4.9% (p value < 0.05) (Figure 2.2A, Table 2.2). Levels of p53 in LN-18 cells were 23±5.4% lower than in T98/EV (p value < 0.05). Overexpression of MGMT (U87/MGMT) did not affect wtp53 or p21 protein levels, compared to its MGMT-negative counterpart empty vector (U87/EV) control (Figure 2.2B). Hence, MGMT silencing was associated with decreased mutp53 protein levels in a GOF mutp53 GBM cell line. Conversely, overexpression of MGMT did not affect p53 levels in wtp53 GBM cells, suggesting that the relationship between MGMT and p53 is restricted to GOF mutp53 context.

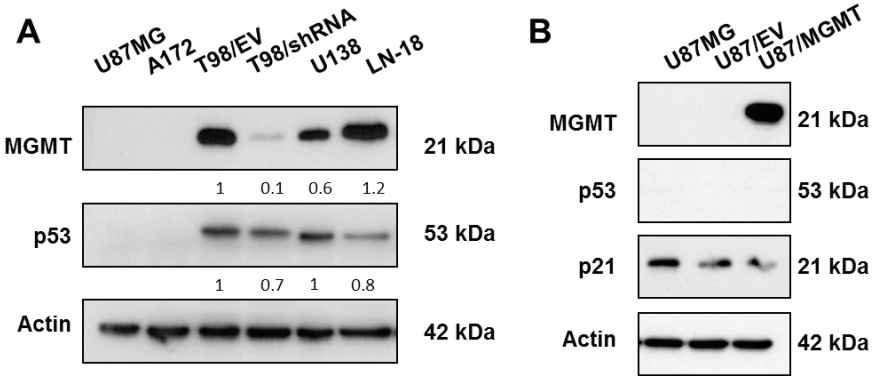


Figure 2.2. MGMT silencing decreased mutp53 protein levels in mutp53 GBM cell lines isogenic for MGMT. (A) Western blotting analysis of the effect of MGMT silencing on expression of p53. Expression of MGMT and p53 in lysates of U87MG, A172, T98G transfected with empty vector control (T98/EV) and shRNA-mediated knockdown of endogenous MGMT (T98/shRNA), as well as U138, and LN-18, A172 and U87MG GBM cell lines. **(B)** Western blotting analysis of expression of MGMT, p53 and p21 in U87MG, U87/EV and U87/MGMT. Actin was used as a loading control. The density of MGMT and p53 bands was normalized to that of T98/EV.

2.4.3 PRIMA-1^{MET} induces cytotoxic effects in GBM cell lines irrespective of p53 status

We used PRIMA-1^{MET} to test the functional consequences of down-regulation of MGMT expression levels in our MGMT isogenic cell lines with GOF mutp53 background. We assessed cytotoxic effects of PRIMA-1^{MET} (24-hour treatment) in GBM cell lines based on MGMT expression and *TP53* status. First, to test the viability of GBM cell lines *in vitro* we treated T98/EV, T98/shRNA, U138, LN-18, U87MG and A172 cell lines with 25, 50, 75 or 100 μM PRIMA-1^{MET} for 24 hours, then cells were kept in drug-free medium for 24 hours (48-hour time point) or 48 hours (72-hour time point). We examined the relative cell number (percentage relative to DMSO control) and viable cell number (% relative to total cell number in each experimental condition) at each time point (24, 48 or 72 hours) using trypan blue exclusion assay and automated cell counting.

The results showed that PRIMA-1^{MET} at 25 μM reduced the relative cell number in T98/EV by 28.8±5.3% at 24 hours, but higher doses were not more effective (Figure 2.3A and Table S2.1). In addition, following drug removal, the cell number was completely restored at 48 and 72-hour time points and was not reduced relative to their respective DMSO controls. By contrast, in T98/shRNA PRIMA-1^{MET} reduced relative cell number in a time and dose-dependent manner (e.g., by 55.5±7.9% and 89.1±1.3% at 50 μM and 100 μM, respectively, at 72-hour time point). The relative cell number decrease in T98/shRNA following 100 μM was significantly greater, compared to that in T98/EV, at all time points (Table 2.3). In U138 cell line, PRIMA-1^{MET} significantly decreased the relative cell number by 37±10.7% at 50 μM and by 59.1±3.1% at 100 μM at 72-hour time point, while in LN-18 the relative cell number was significantly decreased at 100 μM (by 52.1±5.8%), but not at 50 μM (Figure 2.3A and Table S2.1). Treatment with PRIMA-1^{MET} at 50 μM and 100 μM significantly decreased the relative cell number U87MG cell

line by 74.4±3.4% and 88.3±3.9%, respectively, at 72 hours, while in A172 similar doses decreased the relative cell number by 41.5±9.96% and 40.3±4%, respectively.

Table 2.3. Cell number (%) in PRIMA-1^{MET}-treated conditions (100 μM)

Cell line	24 hours		48 hours		72 hours	
	Cell number, % [a]	p-value [b]	Cell number, %	p-value	Cell number, %	p-value
T98/EV	65.6±11.3	-	78.9±11.5	-	113.7±11.2	-
T98/shRNA	15.0±5.0	< 0.0001	26.3±10.7	< 0.0001	11.0±1.3	< 0.0001
U138	74.8±1.6	n.s.	59.7±7.0	n.s.	40.9±3.1	< 0.0001
LN-18	78.3±12.1	n.s.	72.5±10.6	n.s.	47.9±5.8	< 0.0001
A172	57.4±5.0	n.s.	51.4±8.6	0.002	59.7±4.0	< 0.0001
U87MG	55.2±9.7	n.s.	18.7±2.0	< 0.0001	11.7±3.9	< 0.0001

Note: [a] Mean ± SD (relative to DMSO control); [b] Compared to T98/EV at the corresponding time point.

Decreased viability (% of viable cells) was dose-dependent for T98/shRNA, U87MG, A172 and U138 cell lines reaching 18.2±5%, 86.3±10.5%, 26.4±5.7% and 74.6±4.1% decrease, respectively, and only 11.5±10.6% decrease for LN-18 for PRIMA-1^{MET} at 100 μM, 72 hours following treatment (p value < 0.01) (Figure 2.3A and Table S2.1). By contrast, PRIMA-1^{MET} did not induce decreased cell viability in T98/EV up to 100 μM during 72-hour time course. Thus, PRIMA-1^{MET} induced cytotoxicity mostly through reducing cell number in T98/shRNA, U138, LN-18, A172 and U87MG cell lines, but not in T98/EV.

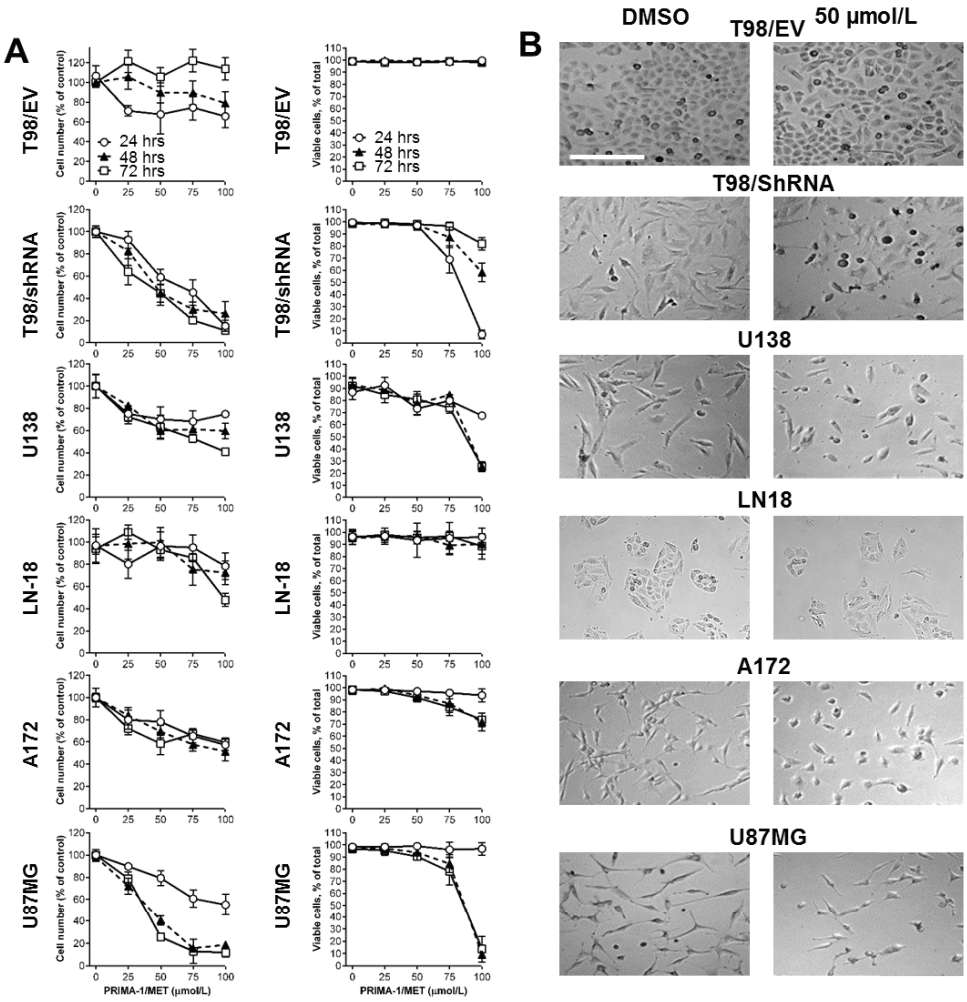


Figure 2.3. PRIMA-1^{MET} reduced relative cell number of GBM cell lines irrespective of p53 status. **(A)** Analysis of the cytotoxic effect of PRIMA-1^{MET} on T98/EV, T98/shRNA, U138, LN-18, A172 and U87MG GBM cell lines using trypan blue exclusion assay and automated cell counting to determine the percentage of relative number of cells in PRIMA-1^{MET}-treated conditions relative to DMSO control at each time point (24, 48 or 72 hours following initiation of a 24-hour treatment with PRIMA-1^{MET}) (left) and the ratio of viable cells (% relative to total cell number in each experimental condition) (right) in the indicated cell lines. Data on graphs represent the mean values ± SD and are representative of at least three independent

experiments. **(B)** Representative micrographs of GBM cells (original magnification 100X) treated with PRIMA-1^{MET} (50 μM, 24 hours) or DMSO control. Scale bar = 250 μm.

Consistent with the quantitative results of the viability assay, the morphological examination showed the predominance of a rounded shape, the presence of sparse and floating cells in T98/shRNA, U87MG and U138, but not T98/EV, A172 or LN-18 cells treated with PRIMA-1^{MET}, compared to their respective controls (Figure 2.3B). Taken together, our results show that PRIMA-1^{MET} preferentially induced time and dose-dependent cytotoxicity mostly through reduced cell number irrespective of p53 status. With the exception of A172, MGMT-negative or low MGMT levels GBM cell lines T98/shRNA, U87MG and U138 were the most sensitive to PRIMA-1^{MET} at all time points.

2.4.4 PRIMA-1^{MET} decreased proliferation and clonogenic potential irrespective of p53 status in GBM cell lines

We further investigated the effect of PRIMA-1^{MET} on proliferation of GBM cell lines using the MTT proliferation assay in GBM cells treated with doses of PRIMA-1^{MET} ranging between 10 and 200 μM. Results of the MTT assay were consistent with viability analysis using the trypan blue exclusion assay. As shown in Figure 2.4A, PRIMA-1^{MET} at 50 μM (corresponding to Log_{10} 1.7 μM on the log scale for the IC_{50} sigmoidal dose-response curve) did not alter proliferation of T98/EV, but inhibited proliferation of T98/shRNA, U138, LN-18, U87MG and A172 cell lines by 28%, 42%, 48%, 30% and 14% (p value < 0.0001), respectively. The IC_{50} for each cell line was as follows: T98/EV - 100 μM, T98/shRNA - 66 μM, U87MG – 60 μM, A172 - 95 μM, U138 – 65 μM, LN-18 – 60 μM. The sensitivity of wtp53 U87MG cells to PRIMA-1^{MET}, which is in the same

range as mutp53 T98/shRNA or U138 suggests that this compound can possibly decrease cell growth independently of p53 status in GBM cells.

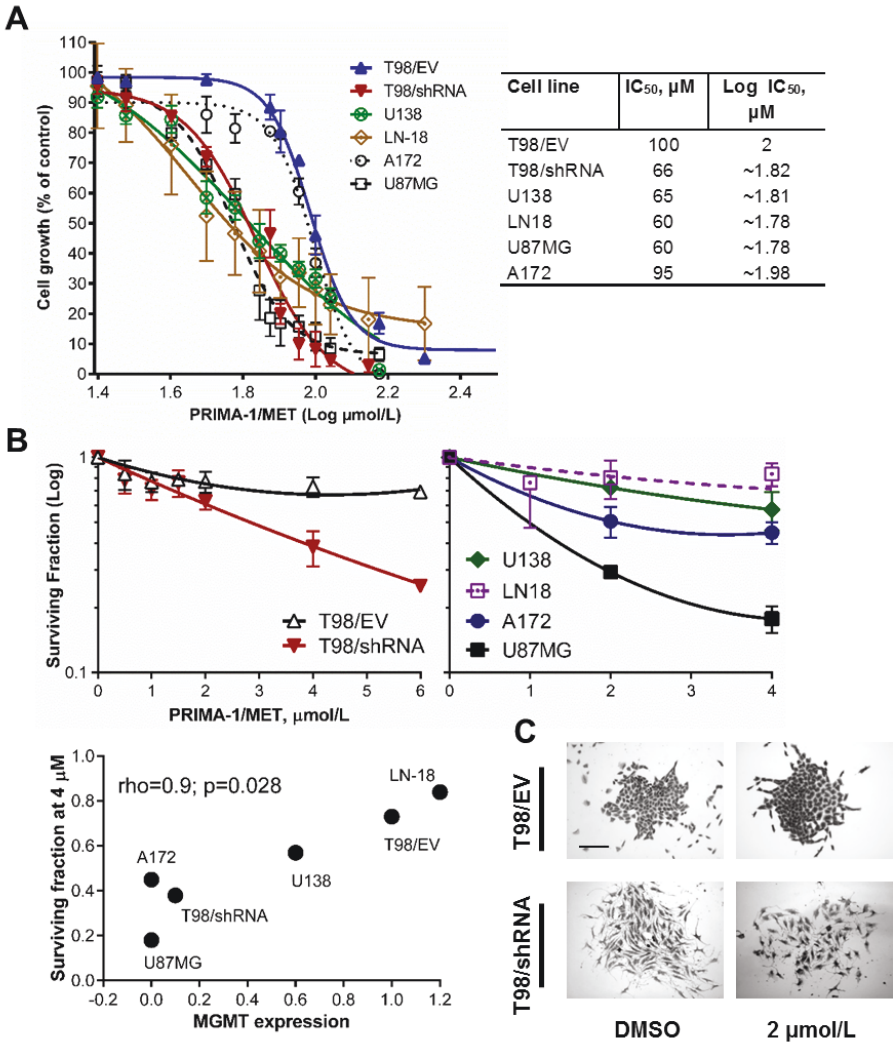


Figure 2.4. PRIMA-1^{MET} decreased proliferation and clonogenic potential of GBM cell lines with different MGMT levels and p53 status. **(A)** Growth-inhibitory effects examined by MTT assay after incubation of T98/EV, T98/shRNA, U138, LN-18, A172 and U87MG GBM cell lines for 24 hours with increasing doses of PRIMA-1^{MET} (10-200 µM) and additional 24 hours in a drug-free medium. Concentration of PRIMA-1^{MET} is on a $\log_{10}$ scale. Graphs represent mean values ± SD from at least three independent experiments performed in triplicate. The resulting

IC_{50} values are shown in the table. **(B)** Colony formation assay results for T98/EV, T98/shRNA (left), LN-18, U138, A172 and U87MG (right) GBM cell lines - the number of colonies (more than 50 cells) was counted and surviving fraction was calculated 8-14 days after treatment with the indicated concentrations of PRIMA-1^{MET} for 24 hours and further incubation in a drug-free medium. Surviving fraction (Y axis, log-scale) was normalized to plating efficiency of the corresponding DMSO controls. Results are means ± SD for at least three independent experiments performed in triplicate. Correlation between MGMT protein levels (from Table 2.2) and surviving fraction of T98/EV, T98/shRNA, LN-18, U138, A172 and U87MG GBM cell lines (bottom row) treated with 4 μM PRIMA-1^{MET}. **(C)** Representative micrographs of T98/EV and T98/shRNA cell colonies stained with methylene blue 7 days following 24-hour treatment with 2 μM PRIMA-1^{MET} (original magnification 100X). Scale bar = 250 μm.

To further explore the cytotoxic effects induced by PRIMA-1^{MET}, we carried out a clonogenic assay to analyze the colony formation ability following treatment of GBM cells with PRIMA-1^{MET}. All cell lines failed to form any colonies at doses higher than 6 μM, suggesting that exposure to PRIMA-1^{MET} for only 24 hours induced long-term cytotoxic effects at lower concentrations than IC_{50}, irrespective of p53 status. The colony-forming ability of T98/EV cells after exposure to PRIMA-1^{MET} at 4 μM was minimally affected and showed a reduction of ~27±7% (p value < 0.0001) (Figure 2.4B). T98/shRNA exhibited a stronger dose-dependent inhibition ~61.7±7.2% at 4 μM (p value < 0.0001). The significant difference in response of T98/shRNA, compared to T98/EV, was detected at a concentration as low as 2 μM (p value < 0.005) and became more drastic with higher concentrations (p value < 0.0001 at 4 μM). The colony formation ability of LN-18 was not significantly decreased (~16.2±10.2% decrease) at 4 μM, but was suppressed

by ~42.8±11.7%, ~57.1±4.7% and ~82.2±2.5% in U138, A172 and U87MG, respectively (p value < 0.001). MGMT protein levels in the tested GBM cell lines significantly correlated with their respective surviving fraction following exposure to 4 μM PRIMA-1^{MET} (n = 6, Spearman's rho = 0.9, p value = 0.028) (Figure 2.4B). Of note, even at a concentration as low as 2 μM, PRIMA-1^{MET} induced spindle-shaped cell morphology and dispersed colonies in T98/shRNA cell line, compared to tight colonies in the DMSO control (Figure 2.4C).

Taken together, our findings suggest that PRIMA-1^{MET} inhibits proliferation and colony-forming potential of GBM cells independently of their p53 status. MGMT silencing caused decreased expression of mutp53 in T98/shRNA cells, which possibly contributes to sensitizing these cells to the anti-proliferative effects of PRIMA-1^{MET}. High levels of MGMT correlate with increased resistance to PRIMA-1^{MET}, while its low levels correlate with increased sensitivity to PRIMA-1^{MET} through long-term effects in GBM cell lines irrespective of their p53 status.

2.4.5 PRIMA-1^{MET}–induced G2/M checkpoint abrogation is associated with MGMT silencing

To further investigate the cell-type-specific effects of PRIMA-1^{MET}, we tested whether the anti-proliferative effect of PRIMA-1^{MET} was mediated by changes in cell cycle progression. GBM cells were treated with a range of PRIMA-1^{MET} concentrations or DMSO and cell cycle distribution was analyzed with propidium iodide staining using flow cytometry (Figure 2.5). Quantification of the percentage of cells in different cell cycle phases indicated that treatment with 25 μM PRIMA-1^{MET} for 24 hours induced a significant increase in a percentage of cells in G2/M phase (from 23.1% to 33.5%) in T98/shRNA compared to DMSO control (data not shown), while 40 μM completely abrogated G2/M checkpoint (Figure 2.5). By contrast, no change was observed after exposure to PRIMA-1^{MET} in T98/EV, confirming the results of cell viability and proliferation

assays. In A172, 40 μM PRIMA-1^{MET} delayed progression through the S-phase (from 21.4% to 37.2%), while in U87MG the cell cycle arrest in G1-phase was detected (from 46.1% to 52.8%) with concomitant decrease in the S-phase. Quantification of cells with sub-G0/G1 DNA content showed that 40 μM PRIMA-1^{MET} induced accumulation of cells in the sub-G0/G1 phase of cell cycle in T98/shRNA (from 0.02% to 16.2%) and to a much less extent in T98/EV and U87MG. Treatment with PRIMA-1^{MET} did not induce changes in sub-G0/G1 population in A172 cells.

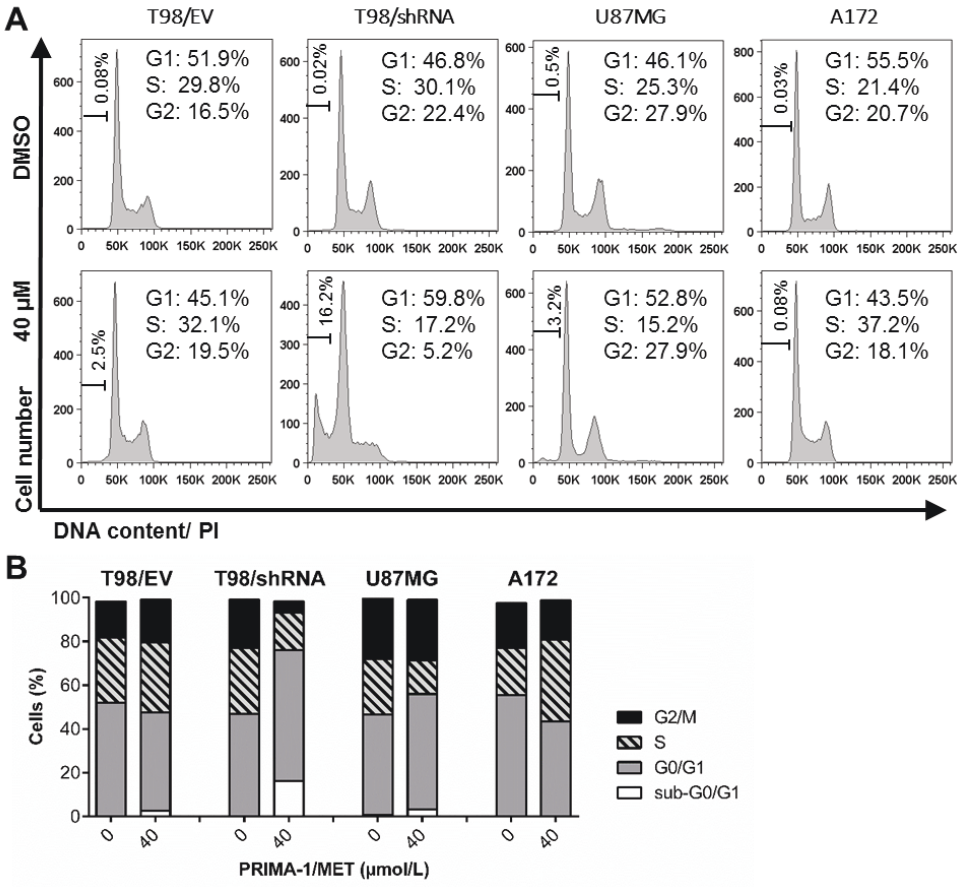


Figure 2.5. PRIMA-1^{MET} induced changes in cell cycle progression in GBM cells with silenced MGMT. (A) Representative histogram plots of cell cycle distribution in T98/EV, T98/shRNA, U87MG and A172 GBM cell lines stained with propidium iodide (PI) at 24 hours following initiation of treatment with 40 μM PRIMA-1^{MET} or DMSO and analyzed by flow cytometry. **(B)** Bar graphs illustrate results of cell cycle analysis shown in (A), indicating the

percentage of cells in sub-G0/G1, G0/G1, S, and G2/M cell cycle phases after treatment with 40 μM PRIMA-1^{MET} or DMSO.

2.4.6 PRIMA-1^{MET} induces dose-dependent decrease of mutp53 protein, increased PARP-1 cleavage and expression of GADD45A in the context of MGMT silencing

To investigate the molecular effects of PRIMA-1^{MET}, T98/EV, T98/shRNA, U87MG and A172 cells were treated using their respective IC_{50} values for 24 hours, lysed and assessed for p53 and MGMT expression using Western blotting. We confirmed decreased p53 levels following MGMT knockdown in T98/shRNA (DMSO control) compared to T98/EV (Figure 2.6A). Strikingly, PRIMA-1^{MET} further suppressed p53 expression in T98/shRNA in a dose-dependent manner. By contrast, PRIMA-1^{MET} treatment did not affect p53 or MGMT expression levels in T98/EV, U87MG or A172 cell lines.

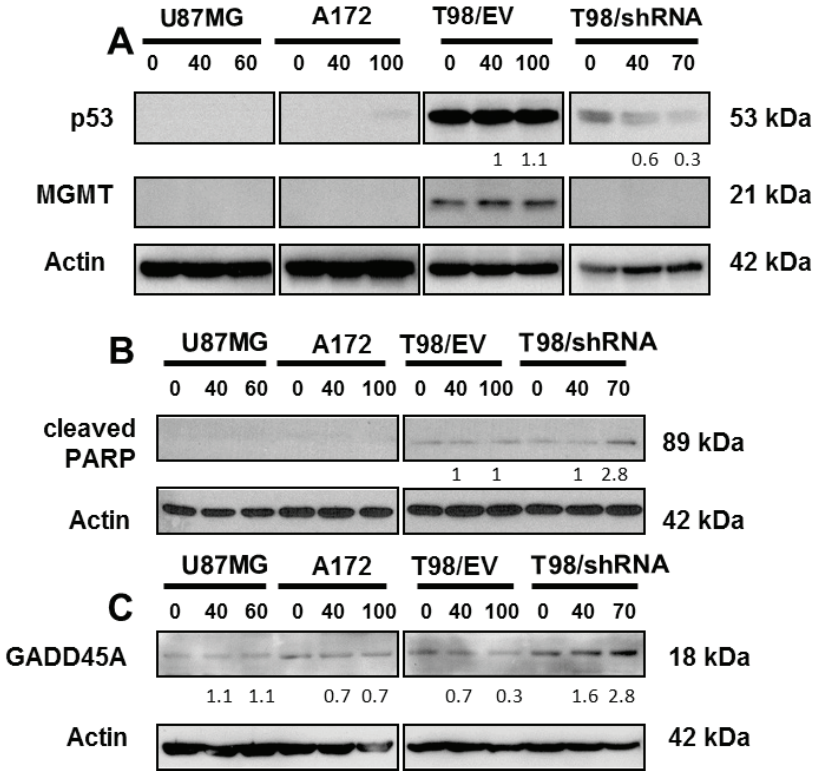


Figure 2.6. PRIMA-1^{MET} decreased expression of mutp53 and increased cleaved PARP-1 and GADD45A in GBM cells with MGMT knockdown. Western blotting analysis of expression of MGMT and p53 **(A)**, cleaved form of PARP-1 (89 kDa) **(B)** and GADD45A **(C)** in U87MG, A172, T98/EV, and T98/shRNA, U87MG and A172 GBM cell lines following 24-hour treatment with DMSO control or PRIMA-1^{MET} using either a common dose for all cell lines (40 μM) or the concentration corresponding to the IC_{50} dose for each cell line. Actin was used as a loading control. The density of the bands was normalized to that of DMSO controls (taken as 100%).

Cleavage of poly(ADP-ribose) polymerase (PARP-1) into fragments of 89 and 24 kDa is a hallmark of apoptosis. Cleaved PARP-1 fragment (89 kDa) was detected by Western blotting in T98/shRNA cells treated with 70 μM PRIMA-1^{MET}, but not in other cell lines (Figure 2.6B), which is in accordance with cell cycle analysis showing the accumulation of T98/shRNA cells in the sub-G0/G1 phase of cell cycle in T98/shRNA.

GADD45A, a DNA damage inducible gene involved in cell cycle arrest and apoptosis is regulated through p53-dependent and independent mechanisms. Interestingly, expression of GADD45A protein increased in T98/shRNA compared to T98/EV. This increase was more pronounced following exposure to PRIMA-1^{MET} (Figure 2.6C) and was maintained up to 48 hours (data not shown). Thus, abrogation of G2 checkpoint and increased sub-G0/G1 cell population detected after PRIMA-1^{MET} treatment is associated with suppression of mutp53 protein expression, increased expression of GADD45A and cleaved PARP-1 in T98/shRNA cells.

2.4.7 PRIMA-1^{MET} induces senescent phenotype in wtp53 U87MG MGMT-negative GBM cell line

To determine the effect of PRIMA-1^{MET} on one of the main p53 targets - cyclin-dependent kinase inhibitor p21, cells were treated by PRIMA-1^{MET} and lysed to assess p21 protein expression by Western blotting. PRIMA-1^{MET} was unable to induce p21 transactivation in GBM cell lines T98/EV and T98/shRNA harboring mutp53 (Figure 2.7A). By contrast, cell lines possessing wtp53, U87MG and A172, showed upregulation of p21 expression upon PRIMA-1^{MET} treatment. Furthermore, U87MG cells treated with as low as 1 μM of PRIMA-1^{MET} exhibited senescent phenotype (Figure 2.7B) as visualized by a positive staining for β-Galactosidase with higher frequency than DMSO control (p value < 0.0001) (Figure 2.7C), while doses above 10 μM led to a massive cell death. By contrast, PRIMA-1^{MET} did not induce senescence in A172, despite elevated p21 levels, or in T98/EV and T98/shRNA (< 0.001% of senescent cells).

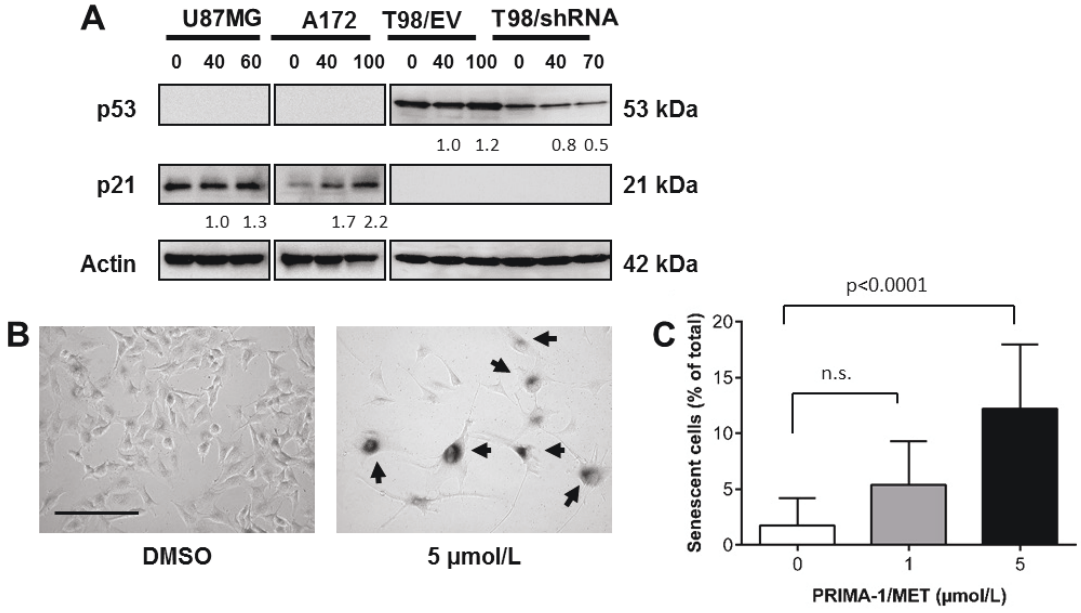


Figure 2.7. PRIMA-1^{MET} treatment increased p21 and senescent phenotype in wtp53 MGMT-negative GBM cells. (A) Western blotting analysis of expression of p53 and p21 in

U87MG, A172, T98/EV, and T98/shRNA, U87MG and A172 GBM cell lines following 24-hour treatment with DMSO control or PRIMA-1^{MET} using either a common dose for all cell lines (40 μM) or the concentration corresponding to the IC_{50} dose for each cell line. Actin was used as a loading control. The density of the bands was normalized to that of DMSO controls (taken as 100%). **(B)** Representative micrographs of senescence-associated β-galactosidase (SA-β-gal)-positive U87MG cells 6 days after the initiation of treatment with 5 μM PRIMA-1^{MET} (original magnification 200X). Arrows show senescent cells. Scale bar = 200 μm. **(C)** Percentage of SA-β-gal-positive U87MG cells 6 days after the initiation of treatment with 1 or 5 μM PRIMA-1^{MET}. Results are means ± SD; total number of cells counted in each condition > 400. P-value for each condition compared to DMSO control is shown; n.s. – not significant.

2.4.8 PRIMA-1^{MET} induces sustained activation of phosphorylated forms of Erk1/2, which is associated with MGMT silencing

Activation of extracellular signal-regulated kinase 1/2 (Erk1/2) has been involved in growth, proliferation, regulation of p53 among other transcription factors, but also in apoptosis [62]. Given the inhibition of proliferation and induction of apoptosis observed following MGMT silencing with PRIMA-1^{MET} treatment, we used Western blotting to assess phosphorylation status (p-Erk1/2) relative to total Erk1/2 as a readout of its activation in U87MG, A172, T98/EV and T98/shRNA cells.

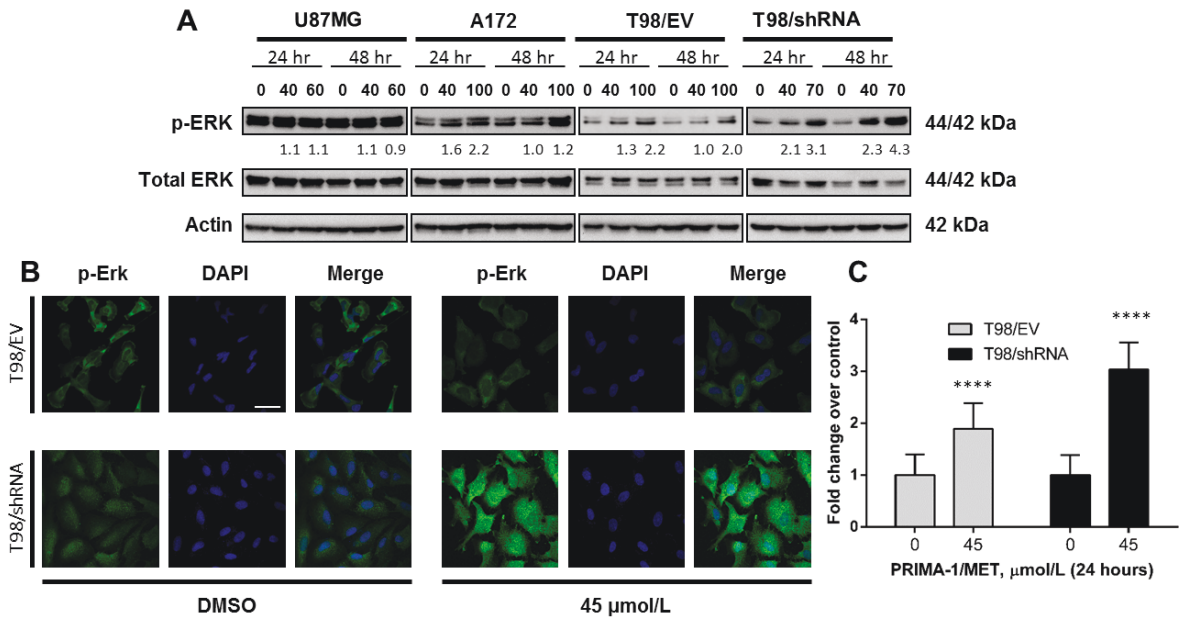


Figure 2.8. PRIMA-1^{MET} modulated expression and distribution of phosphorylated forms of Erk1/2 in GBM cell lines. (A) Western blot analysis showing changes in expression of phosphorylated forms of Erk1/2 (Thr202/Tyr204) in T98/EV, T98/shRNA, U87MG and A172 GBM cell lines at 24 or 48 hours following initiation of treatment with DMSO control or PRIMA-1^{MET} using either a common dose for all cell lines (40 µM) or the concentration corresponding to the IC_{50} dose for each cell line. Actin was used as a loading control. The density of the bands was normalized to that of DMSO controls (taken as 100%). **(B)** Immunofluorescence staining and confocal microscopy analysis of T98/EV and T98/shRNA cells to assess intensity and localization of the phosphorylated forms of Erk1/2 at 24 hours following initiation of treatment with 45 µM PRIMA-1^{MET} (45 µM is ~ IC_{20} for T98/shRNA and < IC_{10} for T98/EV) (original magnification 400X). Scale bar = 50 µm. **(C)** Fold-changes in expression of the phosphorylated forms of Erk1/2 in T98/EV and T98/shRNA GBM cells at 24 hours following initiation of treatment with 45 µM PRIMA-1^{MET} as assessed by immunofluorescent staining using ImageJ software. Results are means ± SD for representative of at least three independent experiments. Total number of cells

analyzed in each condition of experiment > 40 cells. ****, statistically significant difference ($p < 0.0001$) compared to DMSO control.

In U87MG, A172 and T98/EV cells, total levels of Erk1/2 were unchanged with PRIMA-1^{MET} treatment over 24 hours. Interestingly, treatment with PRIMA-1^{MET} induced drastic increase of p-Erk1/2 in T98/shRNA cells (Figure 2.8A), which persisted up to 48 hours following treatment initiation. The expression of p-Erk1/2 was increased to a much less extent in T98/EV and A172 cells, but not in U87MG. Furthermore, fluorescence microscopy showed that PRIMA-1^{MET} did not affect p-Erk1/2 localization in the perinuclear region of T98/EV cells. By contrast, PRIMA-1^{MET} induced a substantial increase in p-Erk1/2 levels and its cytoplasmic localization in T98/shRNA compared to control (Figure 2.8B and 2.8C).

2.4.9 PRIMA-1^{MET} induces cytotoxic effects in GSCs irrespective of p53 status

Given the potential role of GSCs in resistance to treatment and tumor relapse, we further investigated the effect of PRIMA-1^{MET} in GSCs maintained as neurosphere cultures. GSCs were derived from cancer specimens of patients with newly diagnosed GBM as previously described [48]. Western blotting analysis of MGMT protein levels showed that patient-derived GSCs OPK111, OPK161 and 48EF were MGMT-positive, while OPK49 and OPK257 were MGMT-negative. High expression of p53 protein with undetectable or very low levels of p21 evoked mutp53 status for OPK257 (Figure 2.9A). Prospective analysis of p53 by immunohistochemistry confirmed its strong expression in the corresponding patient pathology report (data not shown). Detection of very low levels of p53 protein and basal levels of p21 protein by Western blotting indicate that OPK111, OPK49, OPK161 and 48EF GSCs may display wtp53 function (Figure 2.9A).

We subsequently investigated whether PRIMA-1^{MET} exerts cytotoxic effects in the indicated GSCs. GSCs grown in complete stem cell culture medium were treated with PRIMA-1^{MET} or with vehicle DMSO control for 24 hours, then cells were re-suspended in drug-free stem cell culture medium for a total of 72 hours following PRIMA-1^{MET} or DMSO treatment initiation. We examined the relative cell number (percentage relative to DMSO control) and viable cell number (% relative to total cell number in each experimental condition) at 24 or 72-hour time points using trypan blue exclusion assay and automated cell counting. Exposure to PRIMA-1^{MET} for only 24 hours induced significant time and dose-dependent decrease in the relative cell number in all GSCs even after drug removal (Figure 2.9B and Table S2.2). At doses higher than 20 μM, PRIMA-1^{MET} caused massive cell death with the dominance of cellular debris.

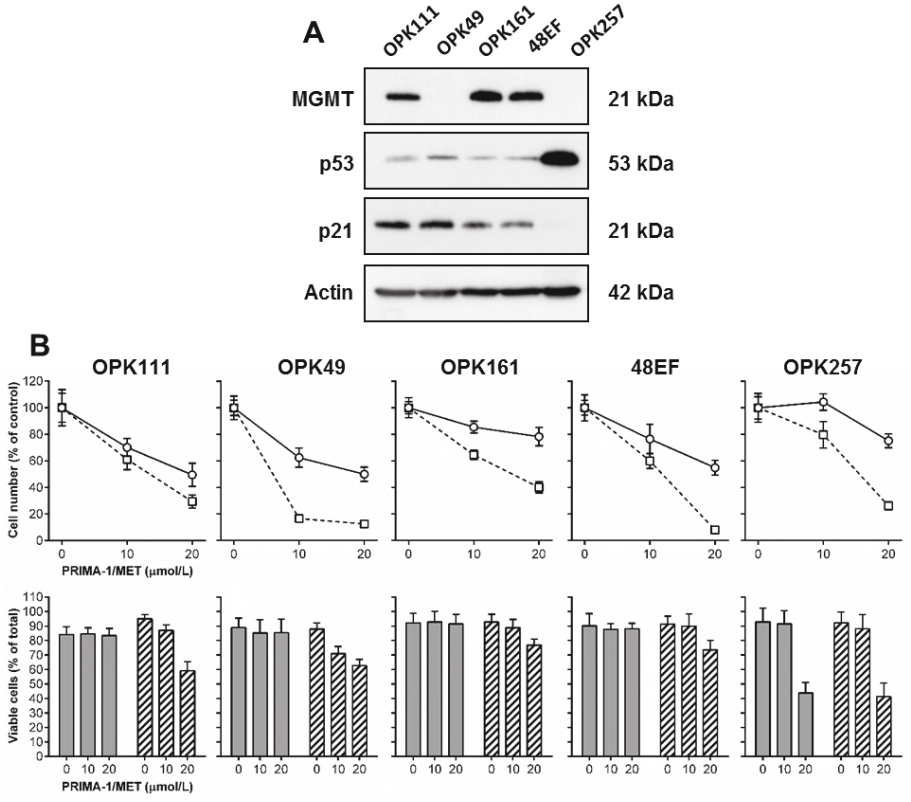


Figure 2.9. PRIMA-1^{MET} decreased relative cell number of GSCs irrespective of p53 status. **(A)** Western blotting analysis showing expression of MGMT, p53 and p21 in OPK111,

OPK49, OPK161, 48EF and OPK257 GSCs. Actin was used as a loading control. **(B)** Analysis of the cytotoxic effect of PRIMA-1^{MET} (10 or 20 μM) on OPK111, OPK49, OPK161, 48EF and OPK257 GSCs using trypan blue exclusion assay and automated cell counting to determine the percentage of relative number of cells in PRIMA-1^{MET}-treated conditions relative to DMSO control at each time point (24 or 72 hours following initiation of a 24-hour treatment with PRIMA-1^{MET}) (top row) and the ratio of viable cells (% relative to total cell number in each experimental condition) (bottom row) in the indicated cell lines. Data on graphs represent the mean values ± SD.

PRIMA-1^{MET} at 20 μM did not induce significant decrease in cell viability (% of viable cells) in either MGMT-positive OPK111, OPK161 and 48EF or MGMT-negative OPK49 GSCs possessing wtp53 at 24 hours (Figure 2.9B and Table S2.2). However, at 72 hours after treatment with 20 μM their viability decreased significantly by 40.9±6.4%, 23.1±4.2%, 26.5±6.4% and 37.4±4.4%, respectively (p value < 0.0001). Similar dose induced 56.3±7.3% and 58.7±9.3% decrease in cell viability in mutp53 MGMT-negative OPK257 at 24 and 72 hours, respectively (p value < 0.0001). Of note, PRIMA-1^{MET} treatment for only 24 hours disrupted the morphology and structure of neurospheres in a dose-dependent manner, and abolished the formation of neurospheres (Figure 2.10).

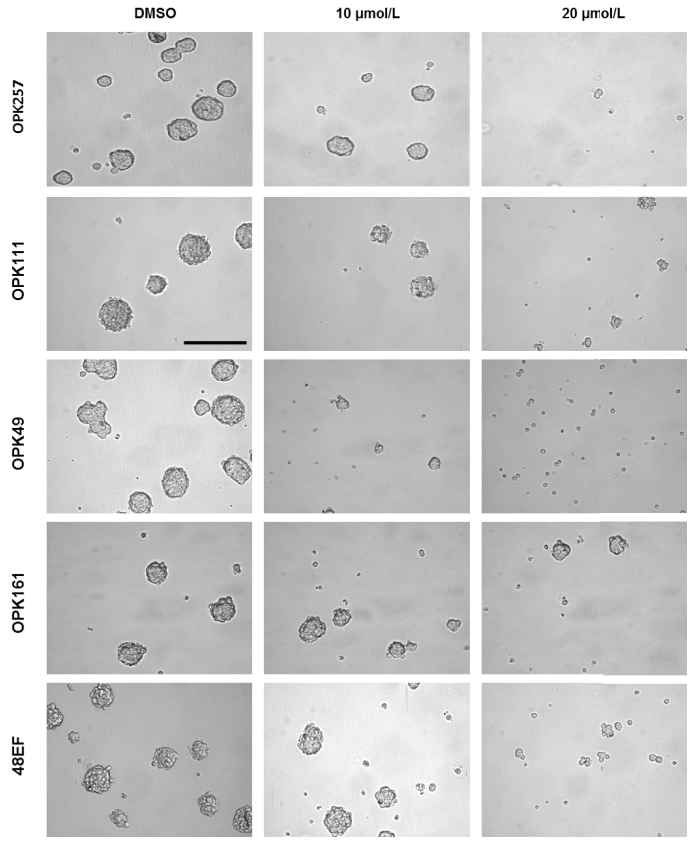


Figure 2.10. PRIMA-1^{MET} decreased relative cell number of GSCs irrespective of p53 status. Representative micrographs of GSCs (original magnification 200X) treated with PRIMA-1^{MET} (10 or 20 µM) or DMSO control at 72-hour time point. Scale bar = 200 µm.

The decrease in viable cell number at 72 hours following the initiation of treatment with 20 µM PRIMA-1^{MET} was also associated with a significant shift in average cell diameter from 12.78±3.3 µm to 11.96±3.4 µm in OPK111, from 14.04±3.9 µm to 10.96±4.3 µm in OPK49, from 14.31±2.94 µm to 12.67±4.96 µm in 48EF and from 15.44±3.6 µm to 11.32±6.0 µm in OPK257 (p value < 0.01), but not in OPK161 (Figure 2.11).

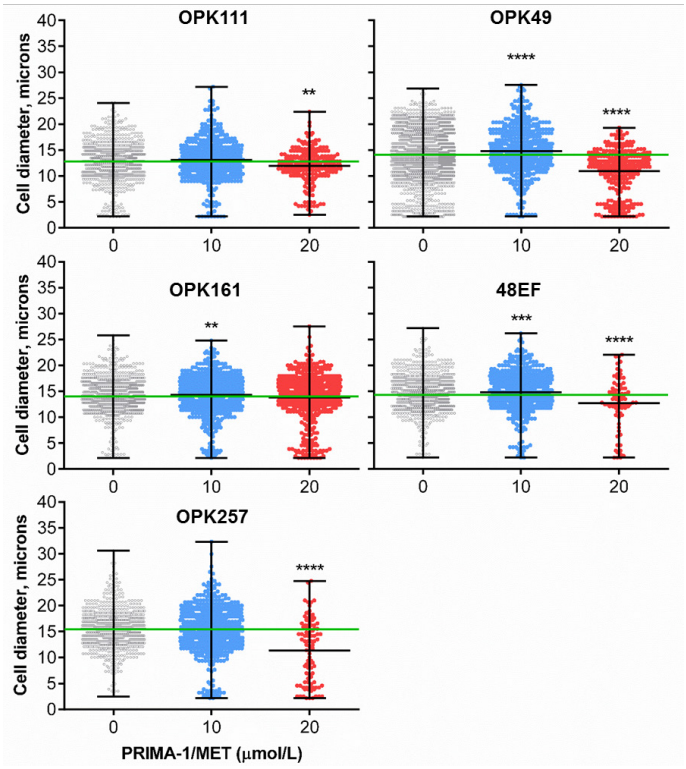


Figure 2.11. Effect of PRIMA-1^{MET} on cell diameter of GSCs. Scatter plots (a range, individual horizontal lines indicate mean) of cell diameter in OPK111, OPK49, OPK161, 48EF and OPK257 GSCs treated with PRIMA-1^{MET} (10 or 20 µM) or DMSO control at 72-hour time point. The common horizontal line indicates mean cell diameter in DMSO control. *, statistically significant difference ($p < 0.05$) compared to DMSO control.

Taken together, PRIMA-1^{MET} decreased relative cell numbers and disrupted the morphology and structure of neurospheres in a time- and dose- dependent manner in both MGMT-positive and –negative wtp53 GSCs at lower doses than in GBM established cell lines. In addition to the aforementioned effects, PRIMA-1^{MET} induced earlier and more pronounced effects on cell viability of mutp53/ MGMT-negative GSC compared to other wtp53 GSCs.

2.4.10 PRIMA-1^{MET} increased wtp53 and decreased mutp53 protein levels with concomitant decrease in MGMT protein levels and activation of Erk1/2 pathway in GSCs

Next, to assess whether PRIMA-1^{MET} affects p53 and MGMT protein levels in GSCs, we analyzed by Western blotting total cellular protein of GSCs lysates following treatment with 20 μM PRIMA-1^{MET} or DMSO control for 24 hours. Interestingly, PRIMA-1^{MET} treatment increased p53 protein with concomitant decrease of MGMT protein levels, compared to DMSO control in wtp53 MGMT-positive OPK111 GSC (Figure 2.12A). There was no further increase of p21 protein (Figure 2.12B). PRIMA-1^{MET} induced a strong activation with increased p53 protein levels and approximately 5-fold increase of p21 protein in MGMT-negative OPK49 GSC. MGMT levels remained undetectable.

PRIMA-1^{MET} did not induce any changes in p53 protein levels, while MGMT levels were decreased in wtp53 MGMT-positive OPK161 GSC. PRIMA-1^{MET} induced activation of p53 without increase in p21 or significant changes in MGMT protein levels in MGMT-positive 48EF GSCs. In sharp contrast, PRIMA-1^{MET} treatment dramatically reduced mutp53 protein levels of MGMT-negative mutp53 OPK257 GSC line. We observed detectable levels of p21 expression in OPK257 treated with DMSO control, which could be mediated through p53-independent pathways. We did not detect caspase-3 or PARP-1 cleavage fragments by Western blotting in GSCs treated by PRIMA-1^{MET} 20 μM for 24 hours (data not shown).

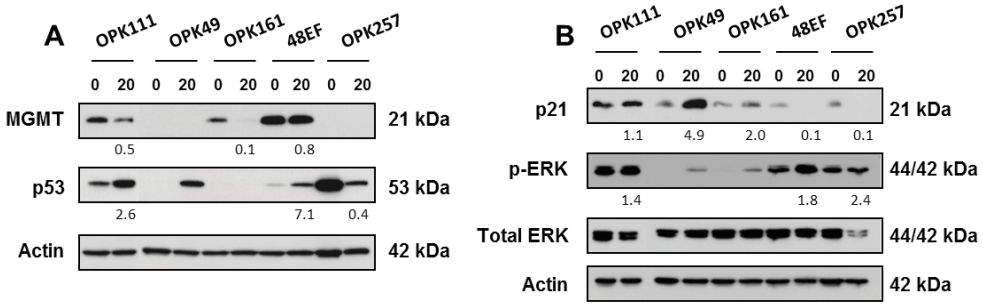


Figure 2.12. PRIMA-1^{MET} modulated expression of wt and mutp53, MGMT, p21 and phosphorylated forms of Erk1/2 in GSCs. Western blotting analysis of expression of MGMT, p53 **(A)**, p21 and phosphorylated forms of Erk1/2 (Thr202/Tyr204) **(B)** in OPK111, OPK49, OPK161, 48EF and OPK257 GSCs following 24-hour treatment with DMSO control or PRIMA-1^{MET} (20 μM). Actin was used as a loading control, p-Erk was normalized to total Erk. The density of the bands was normalized to that of DMSO controls (taken as 100%).

Because PRIMA-1^{MET} treatment for 24 hours increased p-Erk1/2 in A172, T98/EV and T98/shRNA cell lines, we assessed whether PRIMA-1^{MET} induced similar effects in GSCs. Treatment with 20 μM PRIMA-1^{MET} for 24 hours increased Erk1/2 phosphorylation in all GSCs (Figure 2.12B) suggesting that Erk1/2 pathway was activated irrespective of p53 status or MGMT levels. Because of reduced cell number in all GSCs treated with PRIMA-1^{MET}, we could not assess by Western blotting whether Erk1/2 activation was sustained in other time points beyond 24 hours of PRIMA-1^{MET} treatment.

2.5 DISCUSSION

The intricate relationship between p53 and MGMT has not been investigated in light of recent studies highlighting the complex regulation of GOF mutp53 and its activities [20-22]. Because of the low number of GBM cell lines with available information about MGMT and mutp53 RPLA protein levels in the NCI-60 dataset, we investigated the causal relationship between MGMT and p53 using an isogenic pair of mut*TP53* expressing cells with at least 90% knockdown of MGMT. We showed that MGMT silencing decreased mutp53 at the protein level in T98G-based cell model. On the other hand, another study demonstrated that mutp53 knockdown in T98G cells decreased MGMT protein levels, suggesting that mutp53 contributes positively to

MGMT expression [61]. Thus, a potential reciprocal positive relationship between mutp53 and MGMT may uphold the "mutp53/MGMT-positive" phenotype in this model known to harbor GOF mutp53 properties [20]. Previous studies showed that the abundance of mutp53 protein, a hallmark of p53 alterations in cancer, is required for GOF activities such as increased cell proliferation *in vitro* and *in vivo* [21, 63]. Several mechanisms might contribute to the regulation of mutp53 protein levels, such as increased half-life due to the lack of an auto-regulatory loop with the negative regulators MDM2 and MDMX [64], protection of *TP53* gene promoter against repressive histone modifications [65], microRNAs [66] and a transcriptional mechanism via histone deacetylase 8 [67]. Beyond its role as a DNA repair protein, MGMT interacts with >60 MGMT-binding proteins, including several histones and strongly binds to the heat-shock protein 90 (HSP90) [68] known to be involved in protection of mutp53 from ubiquitination [66, 69]. MGMT is also constitutively present at active transcription sites and co-precipitates with the transcription integrator CREB-binding protein CBP/p300 [70], which modulates nucleosomal histones and regulates p53 turnover [71]. The potential relationship between MGMT and mutp53 brings additional piece of evidence for the multifaceted role of MGMT in cancer [47, 70, 72].

We report a causal relationship between expression of MGMT and PRIMA-1^{MET}-induced cytotoxicity through decreased levels of mutp53 protein without restoring wtp53 function in T98G-based model. We showed the convergence of several pathways underlying PRIMA-1^{MET}-induced anti-proliferative and pro-apoptotic effects (Figure 4.1, page 192). Cell exposure to PRIMA-1^{MET} was associated with "loss" of G2 checkpoint and decrease in the S phase population in T98/shRNA. G2/M checkpoint prevents entry into mitosis and its abrogation in the context of MGMT silencing and mutp53 might be an indicator of abnormal response to DNA damage and a mitotic catastrophe, eventually leading to cell death [73]. Indeed, PRIMA-1^{MET} induced increased

ratio of sub-G0/G1 apoptotic fraction and elevated levels of cleaved PARP-1 in T98/shRNA, indicating cell death through apoptosis. Increased susceptibility to apoptotic cell death has been reported in studies using siRNA-mediated knockdown of endogenous mutp53 in different cancer types [74-76]. PRIMA-1, the precursor compound of PRIMA-1^{MET} has been shown to induce nucleolar redistribution of mutp53 associated with p53 degradation *via* ubiquitination as a mechanism that removes the pro-survival function of mutp53 in a breast cancer model [77].

Treatment with PRIMA-1^{MET} increased expression of GADD45A protein in T98/shRNA, but not in T98/EV cells. This is in accordance with studies showing the selective role of GADD45A in the G2/M checkpoint and its function as a tumor suppressor protein through pro-apoptotic and growth suppression activities [78], possibly supported by a mechanism involving GADD45-induced inhibition of the kinase activity of the cdc2/cyclin B1 complex [79]. GADD45A is regulated in both p53-dependent and p53-independent manners. Interestingly, silencing of expression of mutp53 was shown to induce increased expression of wtp53-target genes including *GADD45A* in several human cell lines [74]. Decreased mutp53 levels in T98/shRNA cell line following treatment with PRIMA-1^{MET} could be involved in increased GADD45A.

Several lines of evidence suggest that PRIMA-1^{MET}-induced cytotoxicity was not related to restoration of a wtp53 activity profile. Indeed PRIMA-1^{MET} failed to induce expression of wtp53-target genes, such as p21 for T98-based model. Using the antibody (PAb1620 [29]) that specifically recognizes wtp53 form, we found that PRIMA-1^{MET} did not promote proper folding of the mutant protein in immunofluorescence assays (data not shown). In a previous study using *in vitro* and *in vivo* models of primary and secondary GBM, functional p53-activating signals such as *CDKN2A* (p14^{ARF}) were shown to be required for restoring p53 tumor-suppressor activities following treatment with PRIMA-1 [80]. This is in accordance with our finding showing that

silencing of MGMT in T98G-based model harboring *CDKN2A* mutation and therefore lacking this important functional p53-activating signal, failed to restore wtp53 activity. Thus, restoring wtp53 function and induction of p53 target genes p21, MDM2, and GADD45A through a mechanism involving activation of wtp53 seems to be restricted to *CDKN2A* ($p14^{ARF}$)-competent GBM cells, while selective induction of GADD45A could be achieved in the context of MGMT silencing and decreased expression of mutp53.

Sustained increased levels of phosphorylated Erk1/2 kinases up to 48 hours following treatment of T98/shRNA with PRIMA-1^{MET} is in accordance with a growing number of studies reporting implication of Erk1/2 in promoting cell death through apoptosis in different cancer types [81]. The role of Erk1/2 in apoptosis seems to be cell type specific and also dependent on the levels of its expression, duration of its activity and subcellular localization [82]. The intensity and duration of pro- versus anti-apoptotic signals transmitted by Erk1/2 determines the cell fate towards proliferation or apoptosis. Cytosolic Erk1/2 restrains access to the transcription factor substrates and impedes survival and proliferative signals in the nucleus while increasing the catalytic activity of pro-apoptotic proteins such as death associated protein kinase (DAPK) in the cytoplasm [82].

PRIMA-1^{MET} decreased cell number and suppressed clonogenic capacity of mutp53 U138 cell line expressing intermediate MGMT protein levels to a greater extent compared to T98/EV and LN-18 cell lines. This may reflect recent findings showing the unequal effect of *TP53* mutations, with different mutants displaying a variable profile with respect to loss of wtp53 activity, the ability to inhibit wtp53, and the acquisition of GOF activities [21].

Further investigation of the effects of PRIMA-1^{MET} in established GBM cell lines showed that wtp53/ MGMT-negative U87MG cell line displayed relatively strong basal levels of p21,

heightened sensitivity to PRIMA-1^{MET}, G1/M arrest and was the only cell line undergoing a senescent phenotype in response to PRIMA-1^{MET}. Nonetheless, the senescent phenotype is potentially reversible in p53-intact cells, which may maintain the ability to re-proliferate and escape senescence [83]. By contrast, A172 (heterozygous SNP in p53 proline-rich domain) cell line was resistant to PRIMA-1^{MET}. This could be related to pro-proliferative effects elicited by transient activation of Erk1/2. We also noted a dose-dependent increase of p21 expression without increased p53 levels, suggesting a p53-independent pathway for increased p21. High expression of p21 has been shown to contribute to resistance to drugs through anti-apoptotic effects [84] reported as an "antagonistic duality" of p21 through its role in inhibition of apoptosis [85].

Effects of PRIMA-1^{MET} in both wt and mutp53-harboring cells were reported in different types of cancer. A study conducted by Bao et al. [33] demonstrated that PRIMA-1^{MET} induced p53-dependent apoptotic cell death in wtp53 expressing malignant melanoma cells in 3D culture and in melanoma xenografts *in vivo*. The p53-dependent apoptosis was also triggered by PRIMA-1^{MET} in both mut and wtp53-harbouring Ewing sarcoma cells [86]. The concern that PRIMA-1^{MET} may likely bare toxicity risks for non-cancerous cells, associated with the effects of the drug observed in both wt and mutp53-harboring cells has been addressed in a previous study showing limited cytotoxicity toward normal hematopoietic cells, peripheral blood mononuclear cells and bone marrow mononuclear cells [87]. Potential p53-independent mechanisms of PRIMA-1^{MET}-induced cell death involved ROS and other members of p53 family. PRIMA-1^{MET} toxicity in soft-tissue sarcoma cells was induced through a caspase-independent cell death. ROS-induced toxicity was associated with autophagy induction or JNK pathway activation [88]. Peng et al. [89] demonstrated that PRIMA-1^{MET} inhibited activity of thioredoxin reductase 1, an important regulator of cell redox balance, and, thus, induced cell death through increased oxidation level in

lung adenocarcinoma and osteosarcoma cells irrespective of p53 status. Moreover, PRIMA-1^{MET} was able to restore the pro-apoptotic function to mutp63 and p73 proteins sharing structural homology with p53, in the p53 null lung adenocarcinoma cells stably expressing temperature-sensitive mutant forms of these proteins [90].

To ascertain the potential clinical relevance for the use of PRIMA-1^{MET} in GBM, and because of the important role of GSCs as a disease reservoir in GBM [91], we used patient-derived GSCs with different levels of MGMT and p53 status. Surprisingly, PRIMA-1^{MET} exerted cytotoxic effects in all GSCs at lower concentrations than in established GBM cell lines. The most pronounced early effects on viability (24 hours) were seen in mutp53 MGMT-negative GSC line OPK257, similar to what we observed in T98/shRNA. This supports the general relevance of the effects described in T98/shRNA model and suggests that low levels of MGMT and decreased mutp53 levels correlate with increased cell sensitivity to PRIMA-1^{MET}.

PRIMA-1^{MET} induced activation of wtp53, which was associated with decreased expression of MGMT in MGMT-positive GSCs OPK111. This is in accordance with previous studies showing that wtp53 down-modulates MGMT [22, 23], and a recent study showing that systemic delivery of wtp53 plasmid DNA using an immunoliposome nanocomplex to intracranial GBM tumors decreased MGMT and increased response of TMZ-resistant GBM tumors to TMZ in a mouse model [92]. Additional *in vitro* and *in vivo* studies to assess whether PRIMA-1^{MET} may sensitize TMZ-resistant GSCs through wtp53 activation and decreased expression of MGMT are warranted.

PRIMA-1^{MET} did not upregulate p53, while MGMT was downregulated in MGMT-positive wtp53 GSCs OPK161. This suggests that down-regulation of MGMT could be mediated by p53-independent mechanisms in GSCs. Perhaps, this could be mediated through the JNK pathway, which is critically involved in TMZ resistance and MGMT expression of MGMT-positive GSCs.

Inhibition of JNK, either pharmacologically or by RNA interference in GSCs reduces their MGMT expression and alleviates TMZ resistance [93].

While induction of wild-type p53 protein by some cytotoxic agents often leads to growth arrest and subsequent apoptosis, PRIMA-1^{MET} did not induce PARP-1 or caspase-3 fragments cleavage in GSCs. All GSCs exhibited disruption of neurosphere morphology and structure, cell shrinkage and to some extent lysis of cells with cellular debris evoking necrotic cell death. A similar result was reported for other cell types. PRIMA-1, the precursor compound of PRIMA-1^{MET}, induced necrosis with little apoptosis in mutp53 mouse leukemia L1210 cells [94].

In summary, we provide the first evidence for the convergence of PRIMA-1^{MET}-induced molecular effects leading to activation of wtp53 associated with decreased MGMT protein expression in MGMT-positive GSCs or decreased mutp53 protein levels in mutp53/MGMT-negative cells (i.e., OPK257 and T98/shRNA).

Taken together, our results revealed a potential positive relationship between mutp53 and MGMT in T98G-based model and showed that silencing of MGMT sensitizes GBM cells possessing mut*TP53* to PRIMA-1^{MET}-induced cell cycle arrest and apoptosis. Our findings underscore the cell-context dependent effects of PRIMA-1^{MET} in line with the wide diversity of mutp53 proteins [95] and the steadily evolving list of PRIMA-1^{MET} targets [88-90]. Our study further highlights that the final outcome and the cellular fate following PRIMA-1^{MET} treatment depend on MGMT protein levels and additional cell type-specific factors irrespective of p53 status: *i)* apoptosis in mutp53 GBM cells expressing very low levels of MGMT potentially mediated through abrogation of the G2 checkpoint control, activation of GADD45A and sustained expression of cytoplasmic phosphorylated Erk1/2 kinases (T98G-based model with MGMT silencing) and *ii)* senescence in MGMT-negative GBM cells harboring wtp53 (U87MG).

Future studies need to investigate the role of MGMT as a molecular target for sensitizing GBM cells to PRIMA-1^{MET} and whether PRIMA-1^{MET} may effectively sensitize GSCs to TMZ by decreasing MGMT protein levels. This will provide the proof-of-principle for the potential use of PRIMA-1^{MET} as a strategy to sensitize GSCs through pharmacological depletion of MGMT.

2.6 ACKNOWLEDGEMENTS

The authors thank the managers of the Immunophenotyping (Mrs. Marie-Helene Lacombe, Mrs. Ekaterina Iourtchenko) and Molecular Imaging (Dr. Min Fu) technology platforms of the Research Institute of MUHC for assistance with flow cytometry and confocal microscopy, respectively.

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2.8 SUPPLEMENTAL TABLES

Table S2.1. Relative cell number and viable cells (%) in GBM cell lines treated with a range of PRIMA-1^{MET} doses

PRIMA-1^{MET}, µM	**24 hours**		**48 hours**		**72 hours**	
	Cell number, % [a]	p-value [b]	Cell number, %	p-value	Cell number, %	p-value
	T98/EV					
0	107±9.99	-	100.2±2.3	-	100±5.03	-
25	71.2±5.25	0.0029	105±12.6	n.s.	121±11.5	0.03
50	67.6±19.7	0.0062	89.5±10.1	n.s.	106±9.56	n.s.
75	74.5±12.6	0.0097	89.5±12.1	n.s.	122±11.5	0.009
100	65.6±11.3	0.0017	78.9±11.5	n.s.	113.7±11.2	n.s.
	T98/shRNA					
0	100±5.4	-	100±4.98	-	100±1.91	-
25	92.6±7.7	n.s.	82.5±12.8	0.02	63.9±11.7	< 0.0001
50	59.0±6.95	< 0.0001	44.9±11.4	< 0.0001	44.5±7.87	< 0.0001
75	45.2±11.4	< 0.0001	29.9±6.79	< 0.0001	20.2±1.97	< 0.0001
100	15.0±5.0	< 0.0001	26.3±10.7	< 0.0001	11.0±1.3	< 0.0001
	U138					
0	100±1.8	-	100±10.7	-	100±10.4	-
25	75.1±7.1	0.01	82.4±1.05	0.017	72.3±6.02	< 0.0001
50	70.2±11.3	0.003	59.98±6.7	< 0.0001	63.1±10.7	< 0.0001
75	68.2±9.7	0.001	61.2±1.5	0.0002	52.9±3.4	< 0.0001
100	74.8±1.6	0.017	59.7±7.0	0.0002	40.9±3.1	< 0.0001
	LN-18					
0	97±7.97	-	96.8±14.96	-	93.9±13.2	-
25	80.1±12.7	< 0.0001	98.5±13.7	n.s.	108.9±6.5	< 0.0001
50	96.4±12.6	n.s.	99.8±13.2	n.s.	93.1±7.99	n.s.

75	95.1±11.4	n.s.	75.3±14.3	< 0.0001	85.7±11.6	0.006
100	78.3±12.1	< 0.0001	72.5±10.6	< 0.0001	47.9±5.8	< 0.0001
	A172					
0	100±8.2	-	100±8.6	-	100±3.7	
25	79.95±8.6	0.002	83.9±6.8	0.004	72.2±5.6	< 0.0001
50	78.2±9.97	0.0004	69.4±5.8	< 0.0001	58.5±9.96	< 0.0001
75	65.3±5.9	< 0.0001	57.7±6.0	< 0.0001	67.3±4.5	< 0.0001
100	57.4±5.0	< 0.0001	51.4±8.6	< 0.0001	59.7±4.0	< 0.0001
	U87MG					
0	100±2.8	-	98.2±4.1	-	100±5.0	-
25	89.8±2.1	0.001	71.95±7.2	< 0.0001	78.7±6.1	0.009
50	79.2±6.8	0.0005	40.5±4.3	< 0.0001	25.6±3.4	< 0.0001
75	60.7±7.7	< 0.0001	15.6±2.3	< 0.0001	12.97±10.79	< 0.0001
100	55.2±9.7	< 0.0001	18.7±2.0	< 0.0001	11.7±3.9	< 0.0001
PRIMA-1^{MET}, μM	**24 hours**		**48 hours**		**72 hours**	
	Viable cells, % [a]	p-value [b]	Viable cells, %	p-value	Viable cells, %	p-value
	T98/EV					
0	99±1.4	-	99.3±1.0	-	98.9±0.3	-
25	98.8±1.6	n.s.	99.8±0.5	n.s.	97.99±1.6	n.s.
50	98.3±2.4	n.s.	99±0.8	n.s.	98.7±1.2	n.s.
75	98.6±1.3	n.s.	99.5±0.6	n.s.	99.1±1.0	n.s.
100	99.6±0.9	n.s.	98.5±1.7	n.s.	98.1±1.9	n.s.
	T98/shRNA					
0	99.5±1.0	-	98.4±0.9	-	98.5±0.8	-
25	98.5±1.7	n.s.	98±1.5	n.s.	99.2±0.98	n.s.
50	97±3.9	n.s.	97.3±1.9	n.s.	98.03±2.4	n.s.
75	69±11.3	< 0.0001	87.3±8.4	0.005	96.4±3.4	n.s.
100	7±3.6	< 0.0001	58.3±7.8	< 0.0001	81.8±5.02	< 0.0001

	U138					
0	86.9±6.2	-	92.8±6.5	-	92.1±6.3	-
25	92.5±6.5	n.s.	88.8±0.4	n.s.	84.8±6.6	n.s.
50	73.3±5	0.001	77.6±9.6	0.01	81±4.3	n.s.
75	79.95±5	n.s.	84.6±0.7	n.s.	73.9±4.2	< 0.0001
100	67.5±1.9	0.001	25.2±3.7	< 0.0001	25.4±4.1	< 0.0001
	LN-18					
0	96.3±6.2	-	95.1±5.2	-	95.7±5.4	-
25	96.8±6.6	n.s.	96.3±4.6	n.s.	98±3.1	n.s.
50	93.3±14.1	n.s.	96.9±3.2	n.s.	95.5±5.1	n.s.
75	95.2±12.9	n.s.	89.2±8	< 0.0001	96.7±4.3	n.s.
100	96.1±7.1	n.s.	90.3±8.6	0.0007	88.5±10.6	< 0.0001
	A172					
0	98.3±1.4	-	97.5±1.6	-	98.3±0.9	-
25	98±0.6	n.s.	99.4±0.8	n.s.	97.2±1.3	n.s.
50	97.1±2.2	n.s.	93.6±3.8	n.s.	91.8±3.6	0.031
75	95.7±2.5	n.s.	86.9±7.2	0.0055	83.9±6.9	< 0.0001
100	93.7±5.3	0.027	71.7±7.3	< 0.0001	73.6±5.7	< 0.0001
	U87MG					
0	98.4±1.5	-	96.9±2	-	96.8±3.3	-
25	98.4±1.3	n.s.	97.1±3.6	n.s.	95.4±4.1	n.s.
50	99.03±0.8	n.s.	93.8±6.5	n.s.	90.4±0.2	n.s.
75	96.1±6.1	n.s.	84.5±7.4	0.0027	78.1±11.3	0.0005
100	96.8±5.3	n.s.	9.2±2.0	< 0.0001	13.7±10.5	< 0.0001

Note: [a] Mean ± SD; [b] Compared to DMSO control at the corresponding time point; n.s. – not significant.

Table S2.2. Relative cell number and viable cells (%) in GSC lines treated with a range of PRIMA-1^{MET} doses

PRIMA-1^{MET}, µM	**24 hours**		**72 hours**	
	Cell number, % [a]	p-value [b]	Cell number, %	p-value
	OPK111			
0	100±13.6	-	100±10.8	-
10	70.2±6.7	< 0.0001	61.1±7.7	< 0.0001
20	49.5±8.7	< 0.0001	29.4±4.9	< 0.0001
	OPK49			
0	100±8.8	-	100±5.8	-
10	62.4±7.2	< 0.0001	16.6±2.05	< 0.0001
20	49.97±5.4	< 0.0001	12.4±1.8	< 0.0001
	OPK161			
0	100±7.5	-	100±4.6	-
10	85.5±4.5	< 0.0001	64.6±3.6	< 0.0001
20	78.3±6.9	< 0.0001	40.1±4.2	< 0.0001
	48EF			
0	100±9.8	-	100±5.6	-
10	76.5±11.1	< 0.0001	59.95±5.6	< 0.0001
20	54.8±5.5	< 0.0001	8±1.6	< 0.0001
	OPK257			
0	100±8.3	-	100±10.8	-
10	104.4±6.2	n.s.	79.6±10	< 0.0001
20	75.1±5.2	< 0.0001	26.1±2.98	< 0.0001
PRIMA-1^{MET}, µM	**24 hours**		**72 hours**	
	Viable cells, % [a]	p-value [b]	Viable cells, %	p-value
	OPK111			

0	84.1±5.4	-	95±2.9	-
10	84.6±4.2	n.s.	87±3.7	< 0.0001
20	83.4±4.9	n.s.	59.1±6.4	< 0.0001
	OPK49			
0	89±6.6	-	87.9±4.2	-
10	85.2±9.3	n.s.	71±4.9	< 0.0001
20	85.4±9.4	n.s.	62.6±4.4	< 0.0001
	OPK161			
0	92.2±6.8	-	92.8±5.3	-
10	92.8±7.3	n.s.	88.8±5.9	0.0006
20	91.4±6.6	n.s.	76.9±4.2	< 0.0001
	48EF			
0	90.2±8.4	-	91.4±5.5	-
10	87.8±3.9	n.s.	89.9±8.5	n.s.
20	88.1±3.8	n.s.	73.5±6.4	< 0.0001
	OPK257			
0	92.8±9.6	-	92.2±7.4	-
10	91.5±9.1	n.s.	88.1±9.9	0.039
20	43.7±7.3	< 0.0001	41.3±9.3	< 0.0001

Note: [a] Mean ± SD; [b] Compared to DMSO control at the corresponding time point.

CONNECTING TEXT

In the previous chapter we demonstrated the cell-context dependent effects of PRIMA-1^{MET} p53 targeting compound in GBM cell lines with different expression of MGMT and *TP53* status. In particular, we documented that GBM cells response to PRIMA-1^{MET} is dependent on MGMT levels and occurs irrespective of p53 status. Specifically, MGMT silencing sensitized GOF mutp53 GBM cells to PRIMA-1^{MET}-induced cell death, while MGMT-negative wtp53 U87MG cells developed senescent phenotype following exposure to PRIMA-1^{MET}. In addition, PRIMA-1^{MET} induced decreased MGMT protein expression in MGMT-positive GSCs and decreased mutp53 protein levels in mutp53/MGMT-negative GBM cells (both established and stem cell lines). The content of the previous chapter is covered in the original manuscript recently published in the journal Oncotarget.

These observations directed our interest to the potency of combining PRIMA-1^{MET} with ionizing radiation (IR) - the key component of standard therapy for patients diagnosed with GBM. The radiosensitizing capability of PRIMA-1^{MET} *in vitro* was previously shown in prostate cancer [1], and its synergistic effect with several DNA-damaging compounds was reported in different cancer types [2-4]. In this chapter, we assessed for the first time the potential role of PRIMA-1^{MET} as a radiosensitizer in GBM cell lines with particular emphasis on the effects of PRIMA-1^{MET} pre-treatment and subsequent exposure to IR of GBM cells possessing wt or mutp53 and different MGMT expression levels. Specifically, we describe combined treatment-induced inhibition of cell growth, proliferation and clonogenic potential, in addition to changes in expression of proteins involved in p53, DNA damage and apoptosis pathways in GBM cells. Chapter 3 is reproduced from my manuscript "*In vitro* radiosensitization of glioblastoma cell lines

by PRIMA-1^{MET} (APR-246): cell-context dependent effects based on expression of MGMT and p53 status", which is in preparation for submission to the journal BMC Cancer.

Chapter 3.

IN VITRO RADIOSENSITIZATION OF GLIOBLASTOMA CELL LINES BY PRIMA-1^{MET} (APR-246): CELL-CONTEXT DEPENDENT EFFECTS BASED ON EXPRESSION OF MGMT AND p53 STATUS

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3.1 ABSTRACT

Radioresistance is one of the major factors leading to treatment failure and poor prognosis for adult patients diagnosed with glioblastoma multiforme (GBM), a highly malignant primary brain tumor. Activity of the O6-methylguanine-DNA methyltransferase (MGMT) DNA repair protein and *TP53* mutations account for GBM chemo- and radioresistance. The small-molecule PRIMA-1^{MET}/APR-246 is the first clinical-stage compound able to reactivate mutant (mut)p53 in different cancer types. Given that both p53 and MGMT are involved in DNA repair regulation, we hypothesized that MGMT levels and p53 status of GBM cells may synergistically affect response to p53 targeting by PRIMA-1^{MET} combined to DNA damage caused by ionizing radiation (IR). We analyzed the cytotoxicity of PRIMA-1^{MET} pre-treatment followed by exposure to IR in MGMT-positive mutp53 T98/EV and its isogenic MGMT/shRNA gene knockdown counterpart (T98/shRNA), mutp53 U138 and wtp53 MGMT-negative U87MG and A172 GBM cell lines. Combination of PRIMA-1^{MET} (4 μM) with IR significantly reduced surviving fraction at 2 Gy (SF2) of T98/shRNA and U87MG cells as shown by clonogenic assay. Furthermore, PRIMA-1^{MET} (the concentrations required for 20% or 40% growth inhibition - IC20 or IC40, respectively) combined with IR (2-10 Gy) showed an additive effect or slight to moderate synergism in inhibition of proliferation in all cell lines, except T98/EV. PRIMA-1^{MET} (IC40) with IR induced significantly higher inhibition of the relative cell number than each treatment alone in T98/shRNA and U138 (at 6, 10 Gy for both), U87MG, A172 (2, 4 Gy), but not in T98/EV cells. Interestingly, PRIMA-1^{MET} alone and in combination with IR increased γ-H2AX, pro-apoptotic cleaved PARP and caspase-3 in T98/shRNA and U138, but not in T98/EV cells. By contrast, combined treatment induced senescent phenotype in wtp53 U87MG and A172 cells. Our study shows the cell-context dependent effects of PRIMA-1^{MET} combination with IR, the potential role of MGMT expression

in radiosensitizing effects of PRIMA-1^{MET} for mutp53 GBM cells and provides the basis for investigating these effects using *in vivo* GBM orthotopic models.

3.2 INTRODUCTION

Glioblastoma multiforme (GBM), a grade IV astrocytoma, is the most malignant and common primary brain tumor in adults [5]. Radiation therapy (RT) following surgical tumor resection remains the mainstream treatment modality of therapy for patients newly diagnosed with GBM, despite the high incidence of treatment failure and tumor recurrence due to intrinsic radioresistance [6]. The mechanisms of GBM radioresistance include the increased capability for DNA repair and activation of checkpoint regulators in response to DNA damage [7]. Particularly, glioma cells are frequently characterized by increased levels of DNA repair proteins (e.g. Rad51, DNA-PK) [8], a capability for rapid activation of checkpoint proteins (Chk1 and Chk2) [9] and pro-survival signaling pathways (such as PI3K/Akt) [10]. The status of p53 tumor suppressor protein is another important factor for response of tumor cells to ionizing radiation (IR). Normal functions of wild type (wt)p53 include transactivation of target genes involved in cell cycle arrest, growth control (e.g., p21 – a regulator of G1 checkpoint), induction of DNA repair mechanisms and apoptosis (Puma, NOXA, MDM2, etc.) following exposure to IR [11]. Therefore, mutations of p53, found in about 30% of patients newly diagnosed with primary GBM [12] lead to defective checkpoints that affect response to IR, while mutp53 itself may act in a dominant negative fashion by heterodimerizing with residual wtp53 and blocking its functions [13].

In addition, mutp53 may acquire novel oncogenic functions (gain-of-function, GOF, mutants) [14, 15] leading to resistance to IR via multiple possible mechanisms, such as increased DNA repair efficiency, prevention of apoptosis, altered gene expression and signaling pathways. On the other hand, some high-grade glioma cells expressing mutp53 were shown to repair double

strand breaks (DSBs) caused by IR more slowly and less efficiently compared to wtp53 cell lines, and were, thus, more sensitive to IR [8]. Therefore, although the involvement of p53 in response to IR through defective checkpoints is indisputable, the clear understanding of whether p53 status of tumor cells could be used as an indicator for prediction of their survival following exposure to IR is missing and the results of studies are mixed with conclusions ranging from reduced radiosensitivity of mutp53-expressing cancer cells to no effect of mutp53 status on cancer cell survival or mutp53 associated with increased cell sensitivity to IR [13]. The factors that may explain this controversy include, among others, the differences in genetic background of cancer cell lines (except for isogenic cell lines), type and site of p53 mutation, dose of IR, etc. Additional studies are required to investigate radiosensitization strategies targeting p53-defective checkpoint in GBM cells.

The alkylating agent, temozolomide (TMZ), currently used as a component of standard chemoradiotherapy for GBM patients showed a limited radiosensitizing capability with median overall survival (OS) of 12.1 months with RT compared to 14.6 months with RT+TMZ [16]. Its benefit was rather dependent on tumor promoter methylation of O6-methylguanine-DNA methyltransferase (*MGMT*) with a median OS of 15.3 months for GBM patients with MGMT-negative tumors treated with RT versus 21.7 months with RT+TMZ [17]. MGMT is a small DNA repair protein, which acts by removing methyl adducts from the O(6) position of guanine, thus, interfering with cytotoxicity of alkylating agents, including TMZ. MGMT mRNA expression and activity was also shown to be induced after IR in mouse cell lines *in vitro* and in mouse and rat (liver) tissues *in vivo*, while wtp53 function was suggested to be necessary for such upregulation [18]. Testing the efficacy of TMZ/RT in primary GBM xenografts showed selective radiosensitizing effects only in a subset of MGMT methylated tumors [19]. Hence, both the status

of p53 and expression of MGMT should be taken into account to analyze GBM response to IR and for the identification of chemotherapeutic and targeted agents as potential radiosensitizers in GBM.

PRIMA-1^{MET} (APR-246), a small molecule designed to rescue wtp53 function was shown to selectively inhibit growth and induce apoptosis in mutp53 expressing cancer cells (ovarian, osteosarcoma and lung cancer) *in vitro* and *in vivo* by promoting proper folding of mutp53 and restoration of its normal activity [2, 20-22]. More recently, however, we and others showed the cellular context dependency of PRIMA-1^{MET} cytotoxicity regardless of *TP53* mutational status in GBM [23], prostate cancer and melanoma [1, 24]. Moreover, PRIMA-1^{MET} showed radiosensitizing activity in prostate cancer cells [1] and had synergistic effect when combined with a number of DNA-damaging compounds (e.g. cisplatin, doxorubicin, gemcitabine) in osteosarcoma, NSCLC, ovarian cancer cells, etc. [2-4]. Of note, combination of PRIMA-1^{MET} with platinum-based therapy is tested in phase II study for patients with recurrent p53 mutant high-grade serous ovarian cancer [25].

In this study, we investigated whether pre-treatment of GBM cells with PRIMA-1^{MET} affects their response to IR, while taking into account their MGMT expression and *TP53* status. Hence, we assessed the potential role of PRIMA-1^{MET} as a radiosensitizer using mut*TP53* isogenic cell lines with at least 90% knockdown of MGMT [26], mutp53/MGMT-positive U138 and wtp53/MGMT-negative (U87MG, A172) cell lines. We assessed the potency of PRIMA-1^{MET} combination with IR treatment to suppress their viability, proliferation and clonogenic potential. Analyzing molecular pathways underlying cellular effects of the combined treatment further revealed the cellular context of PRIMA-1^{MET}-induced cytotoxicity and DNA damage independently of restoring mutp53 functions and the optimal potential use of PRIMA-1^{MET} as a radiosensitizer based on MGMT levels and p53 status.

3.3 MATERIALS AND METHODS

3.3.1 Cell culture

The U87MG, T98G, U138 and A172 GBM cell lines were obtained from American Type Culture Collection. T98G-based model described in [26] was used, where cells were transfected with plasmid vector encoding shRNA against MGMT (T98/shRNA) or with empty vector (T98/EV). All cell lines were grown at 37°C in a humidified atmosphere containing 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; standard medium).

3.3.2 Drug treatment and irradiation procedure

Cells grown as monolayers were treated with PRIMA-1^{MET} (Tocris Bioscience, Bristol, UK) dissolved in DMSO at varying doses in standard medium for 24 hours and then left in drug-free medium for additional time depending on the assay used. Cells treated with DMSO were used as a control. Cells pre-treated with PRIMA-1^{MET} or DMSO for 24 hours were irradiated at room temperature using Faxitron x-ray irradiator at a dose rate of 0.648 Gy/min (160 kV; 0.5 mm Cu; 6.3 mA). The irradiation dose varied between 0 and 10 Gy. Corresponding controls were sham irradiated.

3.3.3 Trypan blue exclusion cell viability assay

GBM cell cultures were subjected to varying doses of PRIMA-1^{MET} for 24 hours, then the medium was replaced with a drug-free medium and cells were irradiated as described above. After additional incubation for 48 hours the cell counting was performed using standard protocol of the trypan blue dye exclusion assay in automated Vi-CELL Cell Viability Analyzer (Beckman Coulter,

Inc., Mississauga, ON, Canada) as previously described [23]. Cell number is represented as a percentage relative to cell number in control (100%). Percentage of viable (live) cells is represented in relation to the total cell number in each experimental condition.

3.3.4 MTT assay

Cells were plated in 96-well plates at a density of 2500 cells per well in standard DMEM medium and allowed to adhere overnight at 37°C in 5% CO_2. After that the cells were treated with PRIMA-1^{MET} at varying concentrations for 24 hours, the medium was replaced with a drug-free medium, cells were irradiated as described above and incubated for additional 48 hours before adding 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cell proliferation was measured using Vybrant® MTT Cell Proliferation Assay Kit (Thermo Fisher Scientific Inc.). 10 µl of 0.5% MTT was added to each well in the 96-well plates and 100 µl of 10% sodium dodecyl sulfate (SDS) was added 4 hours after adding MTT. After an overnight incubation, the absorbance was read at 570 nm. To determine whether the type of interaction between PRIMA-1^{MET} and IR was synergistic we used the combination index (CI) method of Chou-Talalay [27] and the CompuSyn software (ComboSyn, Paramus, NJ, USA) [28]. CompuSyn calculates a CI at different concentrations, using the formula for mutually nonexclusive mechanisms: CI = [(D1/Dx1) + (D2/Dx2) + (D1 × D2/Dx1 × Dx2)], where Dx1 and Dx2 are PRIMA-1^{MET} and IR doses, respectively, that are required to achieve a particular fraction affected, and D1 and D2 are the doses of the two agents (combined treatment) required for achieving the same fraction affected. CI > 1 indicated antagonism, CI = 1 indicated additive effect, and CI < 1 indicated synergism. Based on more detailed and refined grading [29], synergism is subdivided into: nearly additive (0.9-1.10), slight synergism (0.85-0.9), moderate synergism (0.7-0.85), synergism (0.3-0.7), strong synergism (0.1-0.3), and very strong synergism (<0.1). Antagonism, respectively, is divided into: slight

antagonism (1.1-1.2), moderate antagonism (1.2-1.45), antagonism (1.45-3.3), strong antagonism (3.3-10), and very strong antagonism (>10).

3.3.5 Clonogenic assay

Cells were plated in 6-well plates, allowed to adhere overnight and treated with PRIMA-1^{MET} at varying concentrations in standard medium for 24 hours. Then the medium was replaced with drug-free medium, cells were irradiated as described above and incubated for additional 7-14 days or until colonies (more than 50 cells) were formed. Cells were then fixed with 10% formalin and stained using 1.5% methylene blue. Colonies of at least 50 cells were counted. The surviving fraction was normalized to the plating efficiency of the corresponding DMSO controls. Sensitizer enhancement ratios (SER_{10} and SER_{50}) were calculated as the ratio of doses of IR required to achieve 10% or 50% surviving fraction for cells without and with PRIMA-1^{MET} [30], using the equation: SERx= Dx(DMSO)/Dx(PRIMA-1^{MET}), wherein Dx is the dose of IR associated with a surviving fraction of x%. Surviving fraction after 2 Gy (SF_2) values were obtained from fitted survival data.

3.3.6 Senescence assay

Cells were stained for senescence-associated beta-galactosidase activity (SA-β-Gal) as described by Dimri et al. [31] using Senescence β-Galactosidase Staining Kit (Cell signaling, Danvers, MA, USA) following the manufacturer's protocol. Briefly, cells were seeded in 6-well plate, allowed to adhere overnight, treated with PRIMA-1^{MET} in standard medium for 24 hours, the medium was replaced with drug-free medium, cells were irradiated as described above and incubated for additional 6 days. Cells were then washed twice with PBS, fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS, and washed twice in PBS. Cells were stained for

24 hours in X-gal staining solution (1 mg/ml X-gal, 40 mmol/l citric acid/sodium phosphate (pH 6.0), 5 mmol/l potassium ferricyanide, 5 mmol/l potassium ferrocyanide, 150 mmol/l NaCl, 2 mmol/l $MgCl_2$). Light microscopy was used to identify senescent (blue stained) cells. The percentage of SA-β-Gal positive cells was quantified by analyzing at least 400 cells in each experimental condition.

3.3.7 Western blot analysis

Cells were washed twice with PBS and lysed with 1X RIPA buffer (Boston BioProducts, Inc., Ashland, MA, USA) supplemented with 0.2 mM sodium orthovanadate, and protease (Sigma-Aldrich, Oakville, ON, Canada) and phosphatase (Roche Diagnostics, Laval, QC, Canada) inhibitors cocktails. Proteins (30 µg, Pierce BCA protein assay kit, Thermo Fisher Scientific Inc.) were electrophoretically separated in 12% SDS-PAGE under reducing conditions and transferred onto PVDF membranes. Membranes were probed for MGMT (Santa Cruz, Dallas, TX, USA), $p21^{Waf/Cip1}$ (Cell signaling, Beverly, MA, USA), mutant and wild-type p53 (DO-1, Santa Cruz, Dallas, TX, USA), β-actin (Sigma-Aldrich, Oakville, ON, Canada), PARP (Cell signaling, Beverly, MA, USA), phospho-histone H2A.X (Ser139, γ-H2AX, 20E3, Cell signaling, Beverly, MA, USA) according to the manufacturer's recommendations. HRP activity was assayed by chemiluminescence using Amersham ECL Western Blotting Detection Reagent (GE Healthcare Life Sciences, Mississauga, ON, Canada). Quantitation of Western blot data was performed using ImageJ software analysis. All data were normalized to loading controls.

3.3.8 Immunofluorescence and confocal microscopy

For immunofluorescence staining, the cells were fixed with 4% paraformaldehyde for 10 min, blocked with 5% normal serum/ 0.3% Triton™ X-100 in PBS for 60 min at room

temperature and incubated with antibody against ɣ-H2AX (Cell signaling) at a working concentration of 0.14 µg/mL, diluted in 1% BSA/ 0.3% Triton™ X-100 in PBS at 4 °C overnight, and then incubated with fluorescence-conjugated secondary antibody Alexa Fluor 488 (Life technologies, Burlington, ON, Canada) at a working concentration of 8 µg/mL diluted in antibody dilution buffer for 60 min at room temperature in the dark. Nuclei were stained with 0.1 µg/mL DAPI (Sigma-Aldrich, Oakville, ON, Canada)). Images were captured (original magnification 400x) using a Zeiss LSM 780 laser scanning microscope (Carl Zeiss MicroImaging, Göttingen, Germany) and analyzed using ImageJ software (>40 cells analyzed in each experimental condition).

3.3.9 Statistical analysis

We used GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) to generate best-fit sigmoidal dose response curves for IC_{50} determination. Data are reported as mean +/- SD and are representative of at least 3 independent experiments unless otherwise stated. Statistics were performed using either an unpaired two-tailed Student's t-test or one-way ANOVA with a post-hoc test as appropriate. P values < 0.05 were considered statistically significant.

3.4 RESULTS

3.4.1 PRIMA-1^{MET} combined with IR induces synergistic cytotoxic effects in GBM cell lines with low MGMT levels irrespective of p53 status

To assess cytotoxic effects of PRIMA-1^{MET} in combination with IR in GBM, we used T98G cell line known to constitutively express high endogenous levels of MGMT and harbor GOF *TP53* mutation [15, 32]. We have previously generated MGMT isogenic cell lines i.e., stable short-hairpin (sh)RNA-mediated 90% knockdown of endogenous MGMT (T98/shRNA) and its

counterpart transfected with empty vector (T98/EV) [26], both possessing p53 mutation in the DNA-binding domain of the protein [23] identical to that previously reported in T98G parental cell line (Table S3.1) [33, 34]. In addition, we used MGMT-positive / mutp53 U138 cell line and MGMT-negative / wtp53 U87MG and A172 (heterozygous single nucleotide polymorphism in proline-rich domain of p53) cell lines.

We have previously analyzed the anti-proliferative effects of PRIMA-1^{MET} on T98/EV, T98/shRNA, U138, U87MG and A172 GBM cell lines using MTT proliferation assay [23] (Table S3.1). To test the effect of PRIMA-1^{MET} combined with IR on their viability, cells were treated with the IC40 dose of PRIMA-1^{MET} or DMSO control for 24 hours, then exposed to 2, 4, 6 or 10 Gy of IR and incubated for a total of 72 hours following initiation of PRIMA-1^{MET} treatment. We examined the relative cell number (percentage relative to the non-irradiated DMSO control) and viable cell number (% relative to total cell number in each experimental condition) using trypan blue exclusion assay and automated cell counting. PRIMA-1^{MET} (IC40, MTT assay) alone did not significantly decrease the relative cell number in mutp53 / MGMT-high T98/EV, while IR alone had dose-dependent effect causing reduction by 28.1±13.2%, 23.7±16.5% and 42.3±13.8% ($p < 0.0001$) following exposure to 4, 6 or 10 Gy, respectively (Figure 3.1 and Table 3.1). The combination of PRIMA-1^{MET} significantly reduced the relative cell number compared to PRIMA-1^{MET} alone only following exposure to IR (6 or 10 Gy) ($p < 0.0001$). By contrast, PRIMA-1^{MET} reduced relative cell number of MGMT-knockdown T98/shRNA cell line compared to DMSO control by 31±6% ($p < 0.0001$), while the effect of IR was dose-dependent (12.3±8.8%, 14.8±9.2% and 32.1±5.3% decrease at 4, 6 and 10 Gy ($p < 0.01$), respectively). PRIMA-1^{MET} combination with IR caused significantly greater decrease in cell number compared to IR alone at IC40 PRIMA-1^{MET} + 2, 4, 6 or 10 Gy ($p < 0.0001$), and compared to PRIMA-1^{MET} alone, at higher IR doses -

IC40 PRIMA-1^{MET} + 6 or 10 Gy ($p < 0.01$). Similarly to T98/shRNA, in mutp53 / MGMT-medium U138 cells PRIMA-1^{MET} alone significantly reduced the relative cell number compared to DMSO control (by 28.3±9%, $p < 0.0001$), while the response to IR was dose-dependent (Table 3.1). The combination of PRIMA-1^{MET} with IR (6 or 10 Gy) was significantly more effective compared to IR alone ($p < 0.0001$) or compared to PRIMA-1^{MET} alone ($p < 0.001$).

For wtp53 / MGMT-negative U87MG cell line, the combination of PRIMA-1^{MET} and IR was significantly more effective for decreasing the relative cell number compared to either IR or drug alone ($p < 0.01$) (Table 3.1), while in A172 the combination led to profound decrease in cell number in comparison with PRIMA-1^{MET} alone ($p < 0.0001$). However, when compared to IR alone, only the combination of PRIMA-1^{MET} with IR at 2 or 4 Gy was significantly more effective ($p < 0.001$) in this cell line.

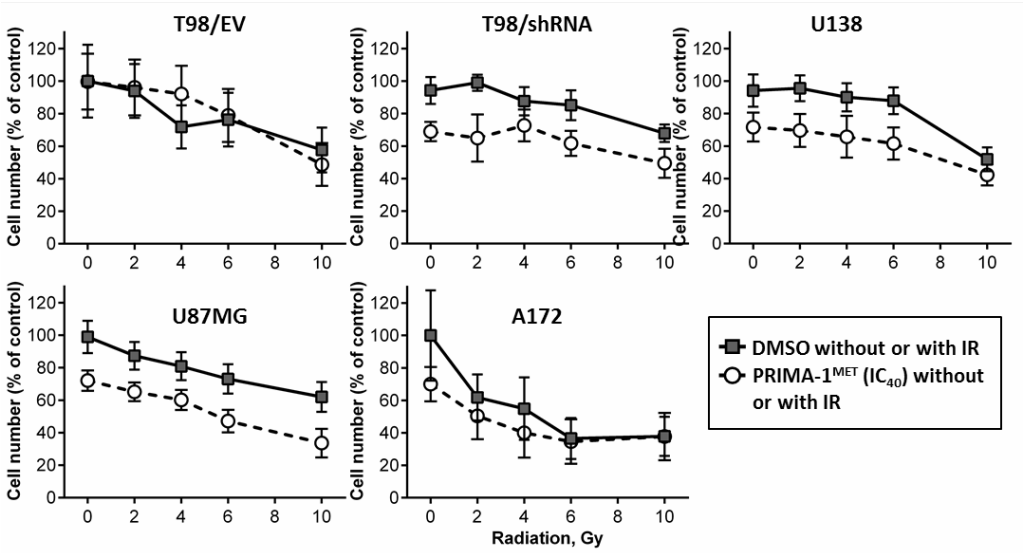

Figure 3.1. Effect of the combination of PRIMA-1^{MET} and IR on the relative cell number of GBM cell lines. Analysis of the cytotoxic effect of PRIMA-1^{MET} (IC_{40}) and a range of IR doses (2, 4, 6 or 10 Gy) on T98/EV, T98/shRNA, U138, U87MG and A172 GBM cell lines

using trypan blue exclusion assay and automated cell counting to determine the percentage of relative number of cells in treated conditions relative to DMSO control in the indicated cell lines. Data on graphs represent the mean values ± SD and are representative of at least three independent experiments.

The viable cell number, in turn, was not significantly affected by PRIMA-1^{MET} (IC40), IR (2-10 Gy) or their combination in any of the analyzed cell lines (data not shown). Thus, PRIMA-1^{MET} (IC40) caused cytotoxicity mainly through decreased relative cell number when used as a single agent in T98/shRNA, U138 and U87MG, but not in T98/EV and A172 cell lines. By contrast, IR alone induced cytotoxic effects through decreased relative cell number in T98/EV, A172 and U87MG, but not in T98/shRNA and U138 cell lines. The combination of PRIMA-1^{MET} and IR was significantly more effective than each treatment alone in mutp53 GBM cells at higher IR doses - T98/EV (10 Gy), T98/shRNA and U138 (6 or 10 Gy), and in wtp53 cells – at all IR doses in U87MG (2-10 Gy) and low IR doses in A172 (2 or 4 Gy) cell lines.

Table 3.1. Relative cell number (%) in GBM cell lines treated with PRIMA-1^{MET} (IC40, μM) and IR (Gy)

	Relative cell number (% of control)													
	Radiation dose (Gy)													
	0 Gy		**2 Gy**			**4 Gy**			**6 Gy**			**10 Gy**		
T98/EV	**Mean±SD***	**p value**	**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**	
0	100±22.3	-	94.0±16.6	n.s.[1]	-	71.9±13.2	<0.0001[1]	-	76.3±16.5	<0.0001[1]	-	57.7±13.8	<0.0001[1]	-
IC_{40}: 93 μM	99.8±17.1	n.s.[1]	96.1±17.2	n.s.[2]	n.s.[3]	92.1±17.3	<0.0001[2]	0.03[3]	78.9±16.3	n.s.[2]	<0.0001[3]	48.7±13.2	0.0012[2]	<0.0001[3]
T98/shRNA	**Mean±SD**	**p value**	**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**	
0	94.3±8.3	-	99.0±4.9	n.s.[1]	-	87.7±8.8	0.007[1]	-	85.2±9.2	0.008[1]	-	67.9±5.3	<0.0001[1]	-
IC_{40}: 59 μM	69.0±6.0	<0.0001[1]	64.9±14.6	<0.0001[2]	n.s.[3]	72.7±9.8	<0.0001[2]	n.s.[3]	61.7±7.7	<0.0001[2]	0.004[3]	49.4±8.9	<0.0001[2]	<0.0001[3]
U138	**Mean±SD**	**p value**	**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**	
0	94.2±9.9	-	95.6±7.9	n.s.[1]	-	90.1±8.7	n.s.[1]	-	87.9±8.2	0.017[1]	-	51.9±7.3	<0.0001[1]	-
IC_{40}: 55 μM	71.7±9.0	<0.0001[1]	69.6±10.2	<0.0001[2]	n.s.[3]	65.8±12.8	<0.0001[2]	n.s.[3]	61.6±9.9	<0.0001[2]	0.001[3]	42.3±6.5	<0.0001[2]	<0.0001[3]
U87MG	**Mean±SD**	**p value**	**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**	
0	99.0±10.0	-	87.3±8.5	0.0001[1]	-	80.9±8.6	<0.0001[1]	-	73.1±9.0	<0.0001[1]	-	62.0±9.2	<0.0001[1]	-
IC_{40}: 54 μM	72.1±6.2	<0.0001[1]	65.2±5.7	<0.0001[2]	0.003[3]	60.2±6.2	<0.0001[2]	<0.0001[3]	47.1±7.0	<0.0001[2]	<0.0001[3]	33.6±8.8	<0.0001[2]	<0.0001[3]
A172	**Mean±SD**	**p value**	**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**		**Mean±SD**	**p value**	
0	100±27.8	-	61.8±14.2	<0.0001[1]	-	54.9±19.2	<0.0001[1]	-	36.5±12.7	<0.0001[1]	-	37.8±12.0	<0.0001[1]	-
IC_{40}: 90 μM	70.0±10.5	<0.0001[1]	50.4±14.3	0.0001[2]	<0.0001[3]	40.0±15.3	<0.0001[2]	<0.0001[3]	34.6±13.7	n.s.[2]	<0.0001[3]	37.7±14.5	n.s.[2]	<0.0001[3]

Note: * Relative cell number (%) normalized to control (mean values ± SD) for each cell line; [1] Relative to non-irradiated DMSO control; [2] Relative to the corresponding dose of IR alone; [3] Relative to PRIMA-1^{MET} (IC_{40}) alone; n.s. – not significant

3.4.2 PRIMA-1^{MET} enhances the IR-induced inhibition of proliferation and clonogenic potential in GBM cell lines

We further investigated the effect of PRIMA-1^{MET} in combination with IR on proliferation of GBM cell lines using the MTT proliferation assay in GBM cells treated with doses of PRIMA-1^{MET} corresponding to IC20 or IC40 for each cell line for 24 hours, followed by exposure to 2, 4, 6 or 10 Gy of IR and incubation for a total of 72 hours following initiation of PRIMA-1^{MET} treatment. As shown in Figure 3.2, IR alone (6 Gy) decreased cell proliferation only by 10% in T98/EV, U138, U87MG and A172 cells and by 15.8±8.3% in T98/shRNA, confirming high radioresistance of the studied GBM cell lines. In T98/EV, compared to PRIMA-1^{MET} alone, the combination of PRIMA-1^{MET} (IC20) and IR caused slightly greater inhibition of proliferation at 4, 6 or 10 Gy ($p < 0.01$), while compared to IR alone - at 2, 4 or 6 Gy ($p < 0.0001$) (Table S3.2). The combination of PRIMA-1^{MET} (IC40) and IR was significantly more effective than IR ($p \leq 0.0001$), but not PRIMA-1^{MET} alone. Based on CI values, the effect of combination PRIMA-1^{MET} and IR was nearly additive or antagonistic in this cell line. In T98/shRNA cells, the combination of PRIMA-1^{MET} (IC20 or IC40) and IR led to significantly greater decrease in proliferation compared to IR alone, but was more effective than PRIMA-1^{MET} alone only at 4 Gy (IC20 or IC40) and at 10 Gy (IC40) ($p < 0.05$). The nearly additive effect occurred in most of cases of PRIMA-1^{MET} and IR combination (CI range = 0.93-1.09) in T98/shRNA cells. As in viability assay, the effect of the combination treatment on proliferation of U138 was similar to than in T98/shRNA, demonstrating significant decrease compared to IR alone (at IC20 or IC40 PRIMA-1^{MET}), while being more effective than PRIMA-1^{MET} (IC20) used as a single agent only at 6 or 10 Gy ($p < 0.05$). This resulted in a moderate (CI = 0.8±0.08 at 6 Gy) or slight (CI = 0.89±0.18 at 10 Gy) synergism in U138. The combination of PRIMA-1^{MET} (IC20 or IC40) and IR in U87MG was significantly more

effective compared to IR alone ($p < 0.0001$), similar to viability results, however, showed no significant decrease in comparison with PRIMA-1^{MET} alone. There was a slight to moderate synergism in all tested combination conditions in U87MG cells (CI ≤ 0.94). Similar to U87MG, the combination was significantly more effective than IR alone in A172. However, in comparison with PRIMA-1^{MET} alone, the combined treatment caused significantly greater decrease in proliferation only at 6 or 10 Gy (IC20), resulting in moderate synergism (CI range = 0.72-0.86).

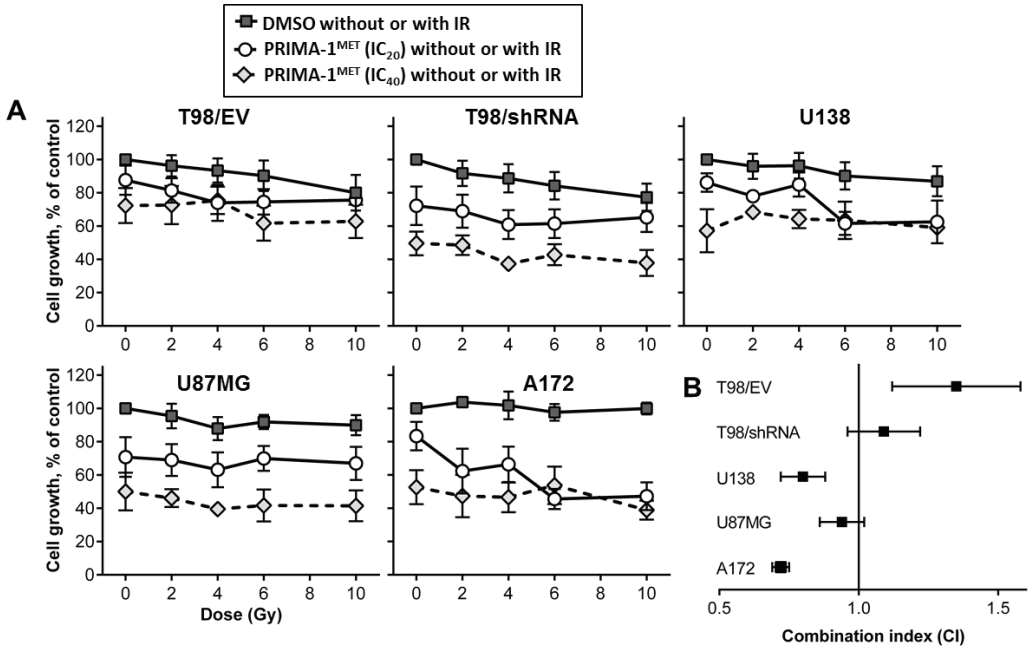

Figure 3.2. Effect of combination of PRIMA-1^{MET} and IR on proliferation of GBM cell lines with different MGMT levels and p53 status. **(A)** Growth-inhibitory effects examined by MTT assay after incubation of T98/EV, T98/shRNA, U138, U87MG and A172 GBM cell lines for 24 hours with concentration of PRIMA-1^{MET} corresponding to IC20 or IC40 value in each cell line or DMSO control, followed by exposure to a range of IR doses and additional 48-hour incubation in a drug-free medium. **(B)** Combination index (CI) values for PRIMA-1^{MET}/ IR (IC20+6 Gy) combinations in T98/EV, T98/shRNA, U138, U87MG and A172 GBM cell lines.

Graphs represent mean values ± SD from at least three independent experiments performed in triplicate.

To further explore the cytotoxic effects induced by the combination, we carried out a clonogenic assay to analyze the radiosensitizing effects of PRIMA-1^{MET} in GBM cell lines. The dose of PRIMA-1^{MET} for combination (4 μM) was chosen based on previous findings that the prolonged incubation (8-14 days) following treatment with higher doses of the drug lead to massive cell death without colony formation in all the studied cell lines [23]. The radiation survival curves showed that the surviving fraction (SF) following exposure to a range of IR doses was decreased when cells were pre-treated with 4 μM PRIMA-1^{MET} in T98/shRNA and U87MG cell lines (Figure 3.3A). The surviving fractions at 2 Gy (SF_2) in these cell lines pre-treated with PRIMA-1^{MET} were significantly lower than in cells treated with DMSO (Figure 3.3B). We also calculated the mean sensitization enhancement ratios (SER) at 10% (SER_{10}) and 50% (SER_{50}) survival as described in Materials and methods. Radiosensitizing effects of PRIMA-1^{MET} with high SER values were observed in T98/shRNA (SER_{50}=2.54; SER_{10}=1.75), U87MG (SER_{50}=2.55; SER_{10}=1.68) and A172 (SER_{50}=2.82) cell lines (Figure 3.3C).

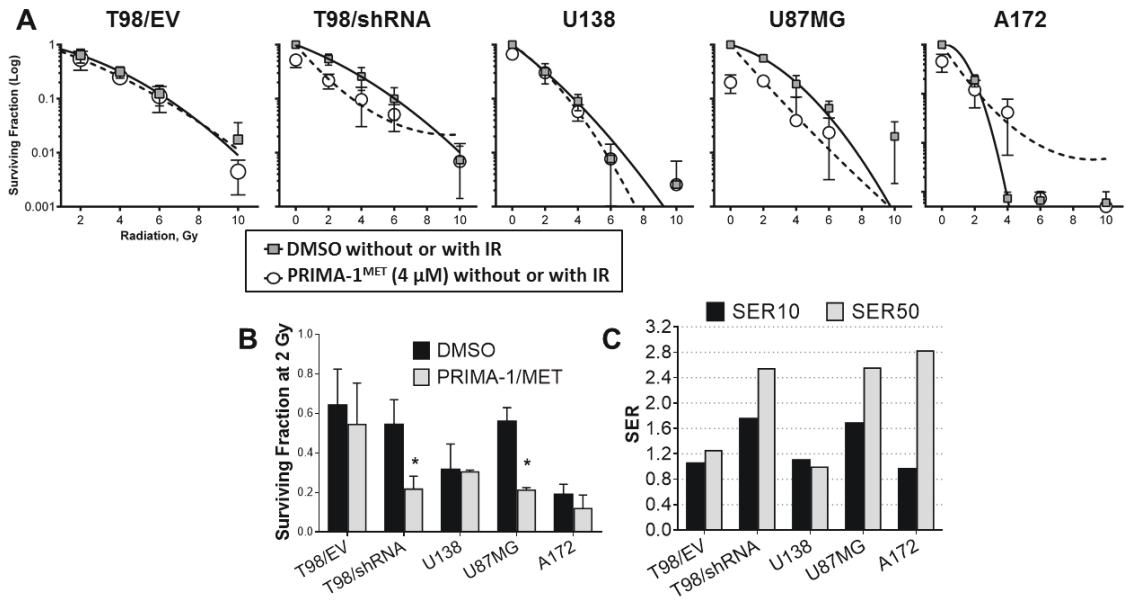

Figure 3.3. Effect of combination of PRIMA-1^{MET} and IR on clonogenic potential of GBM cell lines with different MGMT levels and p53 status. **(A)** Colony formation assay results for T98/EV, T98/shRNA, U138, U87MG and A172 GBM cell lines - the number of colonies (more than 50 cells) was counted and surviving fraction was calculated after treatment with 4 μM PRIMA-1^{MET} for 24 hours followed by exposure to a range of IR doses and further incubation in drug-free medium for 8-14 days. Surviving fraction (Y axis, log-scale) was normalized to plating efficiency of the corresponding DMSO controls. Results are means ± SD for at least three independent experiments performed in triplicate. **(B)** Surviving fraction after 2 Gy (SF2) values obtained from fitted survival data for the indicated cell lines pre-treated with 4 μM PRIMA-1^{MET} or DMSO control. **(C)** Sensitizer enhancement ratios (SER_{10} and SER_{50}) calculated as the ratio of doses of IR required to achieve 10% or 50% surviving fraction for cells pre-treated with DMSO control or 4 μM PRIMA-1^{MET} (SERx= Dx(DMSO)/Dx(PRIMA-1^{MET}, wherein Dx is the dose of IR associated with a surviving fraction of x%).

3.4.3 PRIMA-1^{MET} alone and in combination with IR leads to decreased mutp53, transactivation of wtp53 and p21

To investigate the molecular effects of the combination, T98/EV, T98/shRNA, U138, U87MG and A172 cells were treated with PRIMA-1^{MET} dose corresponding to IC50 value for each cell line for 24 hours and then exposed to 10 Gy. PRIMA-1^{MET} alone and in combination with IR induced decreased levels of mutp53 in T98/shRNA and U138 cells, but not in T98/EV, already at 3 hours post-IR (Figure 3.4A). Pre-treatment of the wtp53 expressing A172 cells with PRIMA-1^{MET} and consequent exposure to 10 Gy induced greater increase in p53 levels compared to IR alone, while none of the treatments was able to activate p53 in U87MG cells. MGMT levels remained unchanged in all cell lines. The expression of one of the main p53 targets - cyclin-dependent kinase inhibitor p21, was not induced following treatment with PRIMA-1^{MET}, IR or their combination in T98/EV, T98/shRNA and U138 GBM cell lines harboring mutp53 (Figure 3.4B). In contrast, wtp53 cell lines showed strong upregulation of p21 expression upon IR or combination treatment in U87MG (at 24-hour time point) and A172 (both at 3 and 24-hour time points).

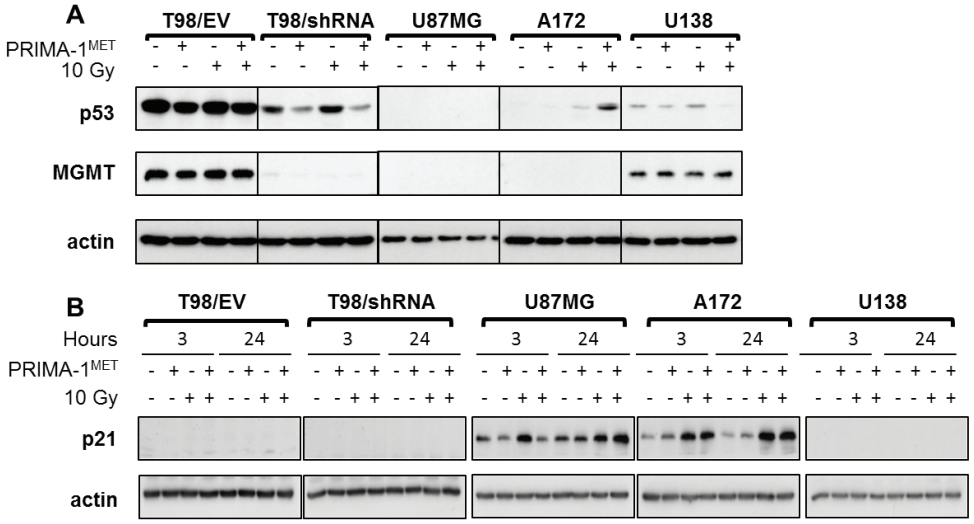

Figure 3.4. Expression of p53, MGMT and p21 following treatment with combination of PRIMA-1^{MET} and IR. Western blotting analysis of expression of p53 and MGMT in T98/EV, T98/shRNA, U138, U87MG and A172 GBM cell lines pre-treated with either DMSO control or PRIMA-1^{MET} concentration corresponding to IC50 value in each cell line and exposed to 10 Gy or sham-control (3 hours post-irradiation) **(A)**, and expression of p21 3 or 24 hours post-irradiation. **(B)**. Actin was used as a loading control.

3.4.4 PRIMA-1^{MET} alone and in combination with IR induces upregulation of proteins involved in apoptosis in GBM cell lines

Cleavage of poly(ADP-ribose) polymerase (PARP-1) into fragments of 89 and 24 kDa is a hallmark of apoptosis. Cleaved PARP-1 fragment (89 kDa) was detected by Western blotting in T98/shRNA, U138, and A172, and to a lesser extent, in U87MG cells treated by PRIMA-1^{MET} alone or in combination with IR and also following exposure to PRIMA-1^{MET} or 10 Gy alone in T98/EV cells (Figure 3.5).

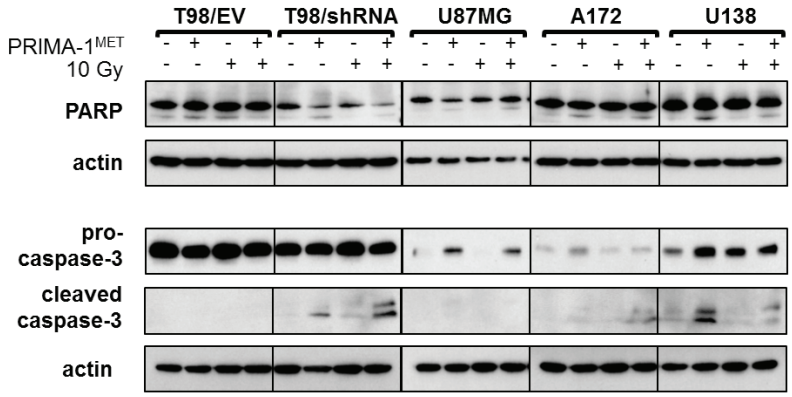

Figure 3.5. Expression of cleaved PARP and caspase-3 following treatment with combination of PRIMA-1^{MET} and IR. Western blotting analysis of expression of total (116 kDa) and cleaved (89 kDa) forms of PARP, pro-caspase-3 and cleaved caspase-3 in T98/EV,

T98/shRNA, U138, U87MG and A172 GBM cell lines pre-treated with either DMSO control or PRIMA-1^{MET} concentration corresponding to IC50 value in each cell line and exposed to 10 Gy or sham-control (3 hours post-irradiation). Actin was used as a loading control.

PRIMA-1^{MET}-induced activation of the intrinsic apoptotic pathway was also evidenced by increased caspase-3 cleavage in T98/shRNA, U138 and to a lesser extent in A172 cells following treatment with PRIMA-1^{MET} alone or in combination with IR (Figure 3.5).

3.4.5 PRIMA-1^{MET} alone and in combination with IR induces increased ɣ-H2AX in mutp53 GBM cell lines

Phosphorylation of H2AX at Ser139, named γ-H2AX, is induced by DNA damage and is used as a hallmark of DNA double-strand breaks (DSBs) [35]. PRIMA-1^{MET} induced increase in the levels of γ-H2AX in mutp53/ MGMT low T98/shRNA and U138 cells to a greater extent than IR alone, but not in mutp53/ MGMT high T98/EV (Figure 3.6A). Treatment with PRIMA-1^{MET} alone or in combination with IR led to similar increase in γ-H2AX in T98/shRNA and U138. A slight increase in γ-H2AX, compared to DMSO control, was detected in A172, but not in U87MG cells following exposure to PRIMA-1^{MET} alone or combined treatment.

The formation of nuclear γ-H2AX foci in T98/shRNA cells following the treatment with PRIMA-1^{MET} alone (40 μM) was confirmed by fluorescence confocal microscopy, while exposure to IR (2 Gy) alone or in combination with PRIMA-1^{MET} induced formation of γ-H2AX foci in both T98/EV and T98/shRNA cells at 3 hours post-IR (Figure 3.6B). The dose of the drug and IR used in immunofluorescence staining experiments had to be decreased compared to Western blotting due to the massive cell death and detachment from the coverslips.

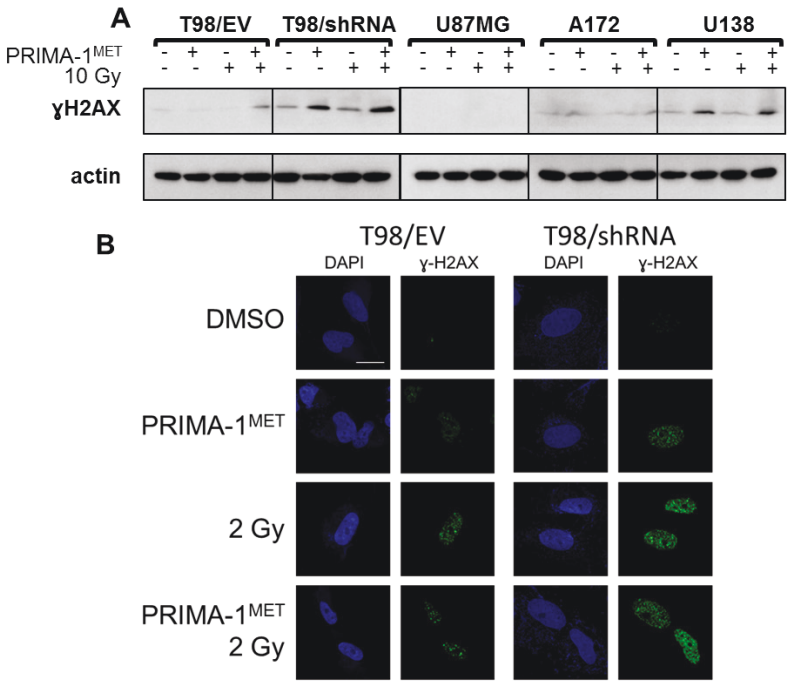

Figure 3.6. Effect of combination of PRIMA-1^{MET} and IR on γ-H2AX levels in GBM cell lines. (A) Western blot analysis showing changes in expression of phosphorylated H2AX (Ser139), γ-H2AX, in T98/EV, T98/shRNA, U138, U87MG and A172 GBM cell lines pre-treated with either DMSO control or PRIMA-1^{MET} concentration corresponding to IC50 value in each cell line and exposed to 10 Gy or sham-control (3 hours post-irradiation) (actin used as a loading control). **(B)** Immunofluorescence staining and confocal microscopy analysis of γ-H2AX foci in T98/EV and T98/shRNA cells pre-treated with 40 μM PRIMA-1^{MET} (<IC20 for T98/shRNA and <IC10 for T98/EV) or DMSO control and exposed to 2 or 0 Gy (3 hours post-irradiation). Original magnification 400X, scale bar = 20 μm.

3.4.6 The combination of PRIMA-1^{MET} and IR induces senescent phenotype in wtp53/ MGMT-negative cells

Given the role of IR in senescence [36] and our own findings showing that PRIMA-1^{MET} induced senescence in U87MG cell line [23], we assessed whether the combination of IR

with PRIMA-1^{MET} affects senescence of T98/EV, T98/shRNA, U138, U87MG and A172 cells lines. Cells were treated with 4 μM PRIMA-1^{MET} for 24 hours and then exposed to a range of IR doses (2-10 Gy) for development of senescent phenotype at 6 days post-IR. In accordance with our previous findings [23], PRIMA-1^{MET}alone induced senescent phenotype with higher frequency than DMSO control in U87MG, but not in A172, as visualized by a positive staining for β-Galactosidase ($p < 0.05$) (Figure 3.7A and 3.7B). IR alone induced senescent phenotype in wtp53 U87MG and A172 cell lines in a dose-dependent manner. U87MG cells pre-treated with 4 μM PRIMA-1^{MET} exhibited senescent phenotype with higher frequency than after IR alone in all tested conditions ($p < 0.05$), while in A172 combined treatment induced significantly higher senescence than IR alone at 6 Gy ($p < 0.05$) (Figure 3.7A and 3.7B). Of note, more than 90% of A172 cells exhibited senescent phenotype following exposure to 10 Gy, irrespective of pre-treatment with 4 μM PRIMA-1^{MET}. By contrast, PRIMA-1^{MET}, IR or their combination did not induce senescent phenotype in mutp53 T98/EV, T98/shRNA and U138 cell lines (data not shown).

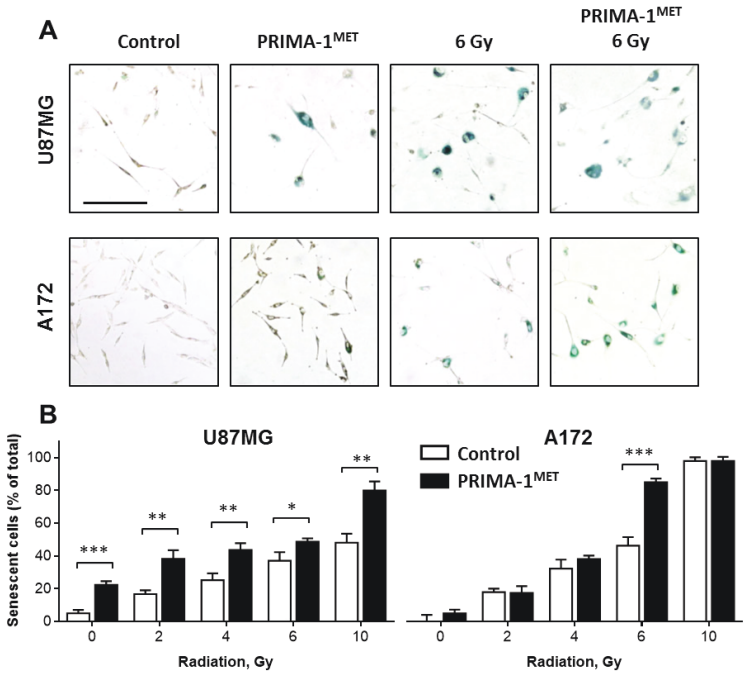

Figure 3.7. Senescent phenotype induced by the combination of PRIMA-1^{MET} and IR. **(A)** Representative micrographs of senescence-associated β-galactosidase (SA-β-gal)-positive (blue) U87MG and A172 cells 6 days after the initiation of treatment with 4 μM PRIMA-1^{MET} (original magnification 200X). Scale bar = 200 μm. **(B)** Percentage of SA-β-gal-positive U87MG and A172 cells 6 days after the initiation of 24-hour treatment with 4 μM PRIMA-1^{MET} or DMSO control followed by exposure to a range of IR doses. Results are means ± SD; total number of cells counted in each condition > 400. P value for each condition compared to IR alone is shown.

3.5 DISCUSSION

Although RT remains a key component of the standard of care treatment for GBM patients, the molecular mechanisms of GBM resistance to IR are still unclear, the prevailing data suggests it is mainly achieved through the ability to augment DNA damage checkpoint activation and increased DNA repair capabilities [7]. MGMT, known to interfere with DNA-damaging activity of alkylating agents (TMZ), and the status of p53 tumor suppressor protein were suggested to be important for cell response to IR, but the role of both MGMT levels and *TP53* status in tumor cell fate is unclear [12, 18, 19].

In this study we investigated the effect of p53-targeting using PRIMA-1^{MET} compound on response of GBM cells to IR. PRIMA-1^{MET}, initially described to restore wild-type function to mutp53 proteins preferentially causing massive apoptosis in mutp53 expressing cancer cells, was later reported to possess p53-independent mechanisms of action in different cancer types [37-39] including GBM as recently shown by our group [23]. As PRIMA-1^{MET} was also shown to enhance

the effect of IR in prostate cancer cells [1], we assessed its potential radiosensitizing capability in GBM cell lines with different MGMT levels and *TP53* status.

We found that 24-hour pre-treatment with PRIMA-1^{MET} preferentially increased radiosensitivity of mutp53 GBM cells with low, but not high, MGMT levels. In particular, PRIMA-1^{MET} enhanced the IR-induced inhibition of relative cell number in U138 and T98/shRNA, but not in T98/EV, while showing a slight to moderate synergy with IR in U138, and additive effect in T98/shRNA cells, but not in T98/EV. Moreover, the ability to form colonies was significantly altered by pre-treatment with PRIMA-1^{MET} prior to exposure to IR, compared to IR alone, in T98/shRNA cells, resulting in a significant decrease in SF2 and in IR dose required to achieve 50% of surviving fraction in this cell line. Treatment with PRIMA-1^{MET} and IR induced increase in pro-apoptotic proteins, cleaved forms of PARP and caspase-3 in T98/shRNA and U138, but not in T98/EV, as shown by Western blotting. These results suggest that PRIMA-1^{MET} not only suppresses the proliferation of mutp53 / MGMT low GBM cells and their ability to form colonies, but is also able to cause cell death through apoptosis.

In accordance with our previous study [23], MGMT silencing decreased mutp53 levels while PRIMA-1^{MET} further induced a drastic decrease in mutp53 levels in T98/shRNA and U138, but not in T98/EV cell line. MGMT and p21 protein levels were not affected in any of these mutp53 cell lines. Importantly, decreased mutp53 levels was previously reported in osteosarcoma [2] and myeloma [40] cell lines and it was suggested that PRIMA-1^{MET} might selectively eliminate cells with high levels of mutp53, while survival of cells with lower mutp53 levels lead to a shift towards the dominance of low mutp53 expressing cells. Expression of mutp53 decreased in T98shRNA but not in T98/EV cell line only 3 hours following exposure to PRIMA-1^{MET}, while exposure to

IR did not affect expression of mutp53. This may suggest a specific role for MGMT in regulation of mutp53 levels in response to PRIMA-1^{MET} at a post-transcriptional level.

PRIMA-1^{MET} alone induced upregulation of ɣ-H2AX in T98/shRNA and U138, but not in T98/EV cells, indicating its ability to induce DNA damage. To our knowledge, this is the first report of PRIMA-1^{MET}-induced formation of ɣ-H2AX foci, which is well recognized as an indicator of DNA DSBs [35]. Increased levels of ɣ-H2AX in T98/shRNA and U138 cell lines is in accordance with previous reports of decreased ability of mutp53 glioma cells to quickly and efficiently repair DSBs [8]. As increased levels of ɣ-H2AX were shown in T98/shRNA and U138, but not in T98/EV cell lines, our study further provides evidence that low levels of the DNA repair protein MGMT might also contribute to the delay in efficient DNA repair. Correlation between MGMT promoter methylation and improved response to radiotherapy was demonstrated in GBM patients [41] and in glioma stem cells (GSCs) *in vitro* [42], while MGMT knockdown in breast cancer cells increased their radiosensitivity and was associated with increased ɣ-H2AX and cleaved caspase-3 [43]. Thus, beyond its role as a DNA repair protein in response to alkylating agents (TMZ) and IR, our findings provide evidence for the role of MGMT in response to PRIMA-1^{MET} and IR, though we cannot conclude whether PRIMA-1^{MET} directly caused DNA damage. Several studies demonstrated the ability of PRIMA-1^{MET} to bind and block the activity of important proteins involved in cellular redox balance, such as glutathione and thioredoxin reductase 1, thus, inducing production of ROS that might lead to ROS-induced DNA damage [38, 40]. Whether ROS may contribute to DSBs formation in GBM cells following treatment with PRIMA-1^{MET} requires further investigation.

In wtp53 MGMT-negative U87MG and A172 GBM cell lines, PRIMA-1^{MET} enhanced the IR-induced decrease in relative cell number similar to mutp53 MGMT low U138 and T98/shRNA

cell lines. Inhibition of colony-forming ability by the combined treatment resulted in a significant decrease of SF2 in U87MG cells and lower IR dose necessary to achieve 50% surviving fraction in both U87MG and A172 cells, compared to IR alone. The radiosensitivity of A172 compared to T98G was previously reported [44]. Expression of p53 was upregulated in A172 after exposure to IR at an early time point (3 hours), while p21 activation was observed in both U87MG and A172 at 24 hours post-IR. Pro-apoptotic protein, cleaved PARP was detected following treatment with PRIMA-1^{MET} alone and in combination with IR in A172 and, to a lesser extent, in U87MG cells, while cleaved caspase-3 was slightly increased following treatment of A172, but not U87MG cell line. Despite the lack of MGMT DNA repair activity in these cell lines, their treatment with PRIMA-1^{MET} combined with IR did not induce DNA DSBs, as γ-H2AX was not upregulated, while apoptosis failed to outcompete senescence as a cell fate decision. Senescence have been demonstrated to occur via either the p53/$p21^{waf1}$ or $p16^{INK4a}$/ Rb pathways [45]. IR-induced senescence was previously reported for U87MG and A172 cell lines [46, 47] and both these cell lines are characterized by $p16^{INK4a}$ deletion [48, 49]. Thus, upregulation of p21 following IR or combined treatment suggests that senescent phenotype may result from the activation of p53/$p21^{waf1}$ signaling in these cells. As previously reported by our group, PRIMA-1^{MET} alone induced senescence with significantly higher frequency compared to DMSO in U87MG, but not in A172 cells [23].

Taken together, our findings demonstrate that potency of PRIMA-1^{MET} as a radiosensitizer in GBM is cell context-dependent. In mutp53 background, PRIMA-1^{MET} enhanced the inhibitory effect of IR on growth and colony formation in GBM cells characterized by low expression of MGMT protein levels, but not in high MGMT-expressing cells. This could be due to the lack of DNA repair activity of MGMT necessary for efficient response to DNA damage caused by

PRIMA-1^{MET} and IR. PRIMA-1^{MET}-induced radiosensitization may occur independently from restoration of mutp53 function, potentially through increased DNA damage (ɣ-H2AX foci) and apoptosis (cleaved PARP and caspase-3). The growth inhibitory effects of combined treatment in wtp53 MGMT-negative GBM cells were associated with activation of p53/p21 signaling and subsequent development of senescent phenotype in IR dose-dependent manner. Additional studies are needed to unravel the exact mechanisms of PRIMA-1^{MET}-induced DNA damage and cytotoxicity independent of restoring mutp53 functions and assess the potential use of PRIMA-1^{MET} as a radiosensitizer, while also considering the role of MGMT in response of GBM cells to the combined treatment.

3.6 ACKNOWLEDGEMENTS

The authors thank the managers of the Molecular Imaging technology platform of the Research Institute of the McGill University Health Centre for assistance with confocal microscopy.

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3.8 SUPPLEMENTAL TABLES

Table S3.1. *TP53* status, p53 and MGMT protein expression and cytotoxic activity of PRIMA-1^{MET} in GBM cell lines isogenic for MGMT and other established human GBM cell lines

Cell line	*TP53* status	Relative p53 protein level[1]	MGMT expression	PRIMA-1^{MET}, μM		
				IC_{20}[2]	IC_{40}	IC_{50}
T98/EV	M237I	1.0	high	81	93	100
T98/shRNA	M237I	0.7±0.49	~90% knockdown	46	59	66
U138	R273H	1.0	medium	37	55	65
A172	R72P heterozygous SNP	<0.1	no	75	90	95
U87MG	wild-type	<0.1	no	43	54	60

Note: [1] Relative p53 protein levels were normalized to β-actin and compared to T98/EV using western blotting and densitometric analysis; [2] Data are IC_{20}, IC_{40} and IC_{50} determined using MTT proliferation assay.

Table S3.2. Effect of PRIMA-1^{MET} and/or IR on proliferation of the indicated GBM cell lines

		Cell growth (% of control)												
		T98/EV												
		Radiation dose (Gy)												
		0 Gy		2 Gy			4 Gy			6 Gy			10 Gy	
PRIMA-1^{MET}, µM		Mean±SD*	p value	Mean±SD	p value		Mean±SD	p value		Mean±SD	p value		Mean±SD	p value	
DMSO		100±2.1	-	96.3±6.4	n.s.[1]	-	93.4±7.2	n.s.[1]	-	90.3±9.2	0.03[1]	-	80.0±10.7	0.001[1]	-
IC20	81	87.6±8.8	0.008[1]	81.4±7.3	<0.0001[2]	n.s.[3]	74.0±10.9	<0.0001[2]	0.002[3]	74.6±7.7	<0.0001[2]	0.0006[3]	75.7±3.7	n.s.[2]	0.0007[3]
IC40	93	72.4±10.5	0.0002[1]	72.7±11.5	<0.0001[2]	n.s.[3]	75.5±8.2	<0.0001[2]	n.s.[3]	61.8±10.5	<0.0001[2]	n.s.[3]	62.9±10.0	0.001[2]	n.s.[3]
		T98/shRNA												
		Radiation dose (Gy)												
		0 Gy		2 Gy			4 Gy			6 Gy			10 Gy	
PRIMA-1^{MET}, µM		Mean±SD	p value	Mean±SD	p value		Mean±SD	p value		Mean±SD	p value		Mean±SD	p value	
DMSO		100±2.2	-	91.7±7.6	0.03[1]	-	88.7±8.5	0.01[1]	-	84.3±8.3	0.0007[1]	-	77.4±8.3	<0.0001[1]	-
IC20	46	72.2±11.6	0.0005[1]	69.0±10.0	<0.0001[2]	n.s.[3]	60.9±8.7	<0.0001[2]	0.04[3]	61.5±8.6	<0.0001[2]	n.s.[3]	65.2±8.7	0.003[2]	n.s.[3]
IC40	59	49.6±7.2	<0.0001[1]	48.6±5.9	<0.0001[2]	n.s.[3]	37.3±3.4	<0.0001[2]	0.002[3]	42.8±6.3	<0.0001[2]	n.s.[3]	37.8±7.8	<0.0001[2]	0.01[3]
		U138												
		Radiation dose (Gy)												
		0 Gy		2 Gy			4 Gy			6 Gy			10 Gy	
PRIMA-1^{MET}, µM		Mean±SD	p value	Mean±SD	p value		Mean±SD	p value		Mean±SD	p value		Mean±SD	p value	
DMSO		100±1.9	-	96.0±7.6	n.s.[1]	-	96.3±7.7	n.s.[1]	-	90.2±8.2	n.s.[1]	-	87.0±9.0	0.02[1]	-
IC20	37	86.2±5.6	0.004[1]	78.0±2.1	0.003[2]	n.s.[3]	85.0±7.3	0.03[2]	n.s.[3]	61.7±6.9	<0.0001[2]	0.004[3]	62.6±12.8	0.0006[2]	0.007[3]
IC40	55	57.3±12.9	0.002[1]	68.4±4.3	0.0002[2]	n.s.[3]	64.3±5.5	<0.0001[2]	n.s.[3]	63.5±11.2	0.0008[2]	n.s.[3]	59.1±4.3	0.0006[2]	n.s.[3]
		U87MG												
		Radiation dose (Gy)												
		0 Gy		2 Gy			4 Gy			6 Gy			10 Gy	
PRIMA-1^{MET}, µM		Mean±SD	p value	Mean±SD	p value		Mean±SD	p value		Mean±SD	p value		Mean±SD	p value	
DMSO		100±2.5	-	95.5±7.4	n.s.[1]	-	87.9±6.9	0.001[1]	-	91.9±4.2	0.0008[1]	-	89.9±6.0	0.002[1]	-
IC20	43	70.8±11.9	0.0008[1]	69.0±9.6	<0.0001[2]	n.s.[3]	63.1±10.5	<0.0001[2]	n.s.[3]	70.0±7.4	<0.0001[2]	n.s.[3]	67.0±10.0	<0.0001[2]	n.s.[3]
IC40	54	50.1±11.3	<0.0001[1]	46.1±5.3	<0.0001[2]	n.s.[3]	39.6±3.6	<0.0001[2]	n.s.[3]	41.7±9.6	<0.0001[2]	n.s.[3]	41.5±9.2	<0.0001[2]	n.s.[3]
		A172												
		Radiation dose (Gy)												
		0 Gy		2 Gy			4 Gy			6 Gy			10 Gy	

PRIMA-1^{MET}, µM		Mean±SD	p value	Mean±SD	p value		Mean±SD	p value		Mean±SD	p value		Mean±SD	p value	
DMSO		100±3.1	-	103.8±1.8	n.s.[1]	-	101.8±8.3	n.s.[1]	-	97.7±5.0	n.s.[1]	-	100±3.6	n.s.[1]	-
IC20	75	83.4±8.6	0.01[1]	62.4±13.4	0.006[2]	n.s.[3]	66.4±10.6	0.002[2]	n.s.[3]	45.6±6.0	<0.0001[2]	0.001[3]	47.3±8.4	<0.0001[2]	0.0005[3]
IC40	90	52.7±10.2	<0.0001[1]	47.4±12.7	0.0003[2]	n.s.[3]	46.6±8.9	<0.0001[2]	n.s.[3]	53.8±11.2	<0.0001[2]	n.s.[3]	38.9±5.7	<0.0001[2]	n.s.[3]

Note: *Cell proliferation rate (%) normalized to control (mean values ± SD) for each cell line; [1] Relative to non-irradiated DMSO control; [2] Relative to the corresponding dose of IR alone; [3] Relative to PRIMA-1^{MET} alone; n.s. – not significant

Chapter 4.

GENERAL DISCUSSION

This PhD thesis describes our novel findings obtained during the investigation of the potential cross-talk between MGMT and p53 in cancer and GBM, in particular, as well as the potency of p53-targeting using PRIMA-1^{MET} compound as a single agent or in combination with IR in GBM cell lines with different levels of MGMT and *TP53* status (wild-type or mutant).

Despite the limited data available about protein levels of MGMT and p53 in public datasets of cancer cell lines and relatively small number of established and stem GBM cell lines used in this study, our results enabled us to contribute to the knowledge about the relationship between MGMT and p53 in GBM and shed light into the potential of optimally using p53-targeting agents while taking into account both p53 status and MGMT protein levels. Previous studies though scarce, were dedicated to investigating the role of p53 in regulating MGMT levels in gliomas and other types of cancer [1-5], while focusing on the potential p53-mediated regulation of MGMT expression and activity, either in a positive or negative manner. Our decision to approach this question with respect to the potential cross-talk between these two proteins, rather than the unidirectional regulation of MGMT by p53, was based on the recent reports from our group [6, 7] and others [8, 9] suggesting additional functions of MGMT beyond its known role as a protein repairing the mutagenic DNA lesion (O6-methylguanine) introduced by alkylating agents (TMZ), thus, interfering with their cytotoxicity. Due to the complex transcriptional and translational regulation mechanisms of MGMT and p53 [10-15], we were interested in assessing the characteristics of p53/MGMT relationship on both mRNA and protein levels and not solely in GBM, but also in cell lines representing other cancer types in publicly available datasets. The findings of this part of the study are too fresh and have a number of limitations to provide the full picture of the mechanisms underlying the cross-talk between p53 and MGMT which need to be further investigated at the transcriptional, post-transcriptional and translational levels while taking

into account the complex molecular heterogeneity of GBM. Our exploration also focused on investigating the response of GBM cells characterized by different MGMT expression levels and *TP53* status to p53 targeting by PRIMA-1^{MET} compound alone or in combination with IR. This underexplored area of research is characterized by limited data about the role of p53 status and MGMT levels in response to radiotherapy [16, 17], with thus far only two reports on mutant p53 targeting [18, 19] in addition to a relatively new field of investigation for the effects of PRIMA-1^{MET} in GBM. Our findings hopefully provided preliminary insights into the molecular alterations underlying the growth inhibitory effects induced by PRIMA-1^{MET} ± IR, leading to differential cell fate decisions (apoptosis or senescence) based on MGMT and p53 in GBM cell lines. While our study is the first to assess the efficacy of this compound in GBM while taking into account MGMT protein levels, it could not reveal all the aspects of PRIMA-1^{MET} mechanism of action and potency as a radiosensitizer. We hope that our promising findings will serve as a basis for future *in vitro* and *in vivo* studies to fully characterize the mechanisms of PRIMA-1^{MET} cytotoxicity and provide the proof-of-principle for its potential use as a component of therapy for patients with GBM and possibly other types of cancer.

4.1 THE RELATIONSHIP BETWEEN MGMT AND p53 IN CELL LINES FROM PUBLICLY AVAILABLE DATABASES AND GOF MUTP53 GBM CELL LINE

MGMT and p53 proteins are involved in DNA repair pathways and play important roles in GBM tumor response to chemo- and radiotherapy. Wild-type p53 was suggested to regulate MGMT expression [1-3, 20], while the lack of MGMT activity was associated with *TP53* G:C to A:T transition mutations in different types of cancer [21-29]. In this study we explored the novel

concept of the reciprocal relationship between MGMT and p53 in cell lines from different cancer types and GBM, in particular. We sought to explore the potential interrelation between MGMT and p53 using data from publicly available cell lines datasets and GBM cells isogenic for MGMT and possessing wt or mutp53.

Our finding of a positive correlation between mRNA levels of MGMT and wtp53 in glioma cell lines based on CCLE data analysis described in Chapter 2 is in contrary with previous studies suggesting wtp53-mediated inhibition of MGMT mRNA expression in glioma *in vitro* [1, 2]. This discrepancy may be because expression of mRNA does not always reflect protein levels, especially for genes known to be tightly regulated at the post-transcriptional level, such as *TP53* [15] and *MGMT* [11-13]. At the protein level, analysis of NCI-60 cell lines across tumors derived from 9 different tissues revealed a strong negative correlation between MGMT and mutp53. We could not conduct the statistical analysis of MGMT and p53 protein levels within GBM subgroup due to a small number of available cell lines.

Previous reports showed a significant correlation between low MGMT and mut*TP53* status of tumor cells. Specifically, *MGMT* promoter methylation, a major mechanism of MGMT silencing was correlated with mut*TP53* status of tumor cells in tissue samples obtained from patients with GBM, low grade glioma, anaplastic astrocytoma, colorectal and lung cancer [21-25]. Similarly, low MGMT levels or activity were shown to be associated with mutp53 status in tissue samples from patients diagnosed with GBM, astrocytomas or primary breast cancer [26-30]. Using GBM cell lines isogenic for MGMT and GSCs, our study extends the results on association between low MGMT and mutp53 status by revealing a positive correlation between the levels of these two proteins. Our study results also corroborate the suggested model [27] of how GBM tumors might develop a low MGMT/ mutp53 profile: *i)* initial upregulation of wtp53 during early

phase of GBM development; *ii)* wtp53-mediated downregulation of MGMT expression [1-3, 20]; *iii)* MGMT silencing by gene promoter methylation during GBM progression; *iv)* gradual accumulation of p53 mutations in GBM tumors in absence of MGMT activity [21-29]. Finally, as mutp53 may confer novel oncogenic properties (GOF) [31-33], the tendency toward low MGMT/ high mutp53 profile might bear functional significance for increased resistance of such tumor cells to chemo- and radiotherapy.

To examine the MGMT / p53 relationship in GBM cells *in vitro*, we employed two isogenic models: GOF mutp53 [32] T98G-based cell model with at least 90% knockdown of MGMT (T98/shRNA) [7] and wtp53 U87MG-based model with MGMT overexpression (U87/MGMT). An important observation was that MGMT silencing decreased mutp53 at the protein level in T98/shRNA, while MGMT did not affect wtp53 expression in U87/MGMT. Our finding extends the results of the study showing that mutp53 knockdown induced decrease in MGMT protein levels in T98G cells [34] suggesting a potential reciprocal positive relationship between mutp53 and MGMT in this model known to harbor GOF mutp53 properties. This is in accordance with studies showing that MGMT binds multiple proteins, including those involved in regulation of p53 turnover [8, 9, 35-37]. Our findings provide evidence for the hypothesis that beyond its well-known DNA repair function, MGMT is involved in other cellular processes and has a relevant multifaceted role in cancer.

The results of this part of our study reveal the potential relationship between MGMT and mutp53 in GBM and, at the same time, raise intriguing questions, which require further investigations. Particularly, the validation of NCI-60-based findings in a larger panel of cell lines will provide better understanding about whether the negative correlation between MGMT and mutp53 holds true for all cancer types or is a distinct feature of some of them. The modulation of

MGMT levels, possibly with O6-BG pseudosubstrate, will allow to conclude whether the suggested potential reciprocal relationship between MGMT and GOF mutp53 is also observed in mutp53 GBM models other than T98G-based one, used in our study. Ultimately, the validation of *in vitro* findings in tumor tissue samples obtained from patients with GBM and other cancer types (e.g., using TCGA dataset) will provide insight on the nature of MGMT/p53 relationship, particularly in different molecular and epigenetic subtypes (GBM subtypes reported in [38-40]), and determine its prognostic and predictive relevance with regards to clinical parameters (response to therapy, OS, PFS, etc.). Answering all these research questions undoubtedly will not be easy and will require overcoming a number of challenges, such as development of the standardized methodology for MGMT and p53 protein levels assessment in tissue samples (IHC, MGMT activity assays) and coping with numerous types of p53 mutations, including those with GOF activities.

4.2 THE ROLE OF MGMT LEVELS AND *TP53* STATUS IN GBM CELLS IN RESPONSE TO PRIMA-1^{MET}

Due to the important role of p53 in tumor response to chemo- and radiotherapy, multiple strategies to target p53 have been developed, such as TP53-based gene therapy, p53 vaccines, agents for prevention or disruption of p53-MDM2 interaction, and small compounds for rescue of mutp53 [41]. Only two previous studies tested mutant p53 targeting agents in glioma *in vitro* [18, 19]. In this part of the study (Chapter 2) we assessed for the first time the effects of PRIMA-1^{MET}, which is at the clinical stage of development [42, 43], in a panel of established GBM cell lines and GSCs obtained from patients newly diagnosed with GBM. Given our findings on MGMT/p53 relationship we sought to characterize the role of MGMT expression and p53 status of GBM cells in their response to PRIMA-1^{MET}.

Although PRIMA-1^{MET} was originally selected based on the ability to selectively target mutp53 expressing cell lines from different cancer types [44], our observation of PRIMA-1^{MET}-induced cytotoxicity in GBM cells irrespective of their p53 status agrees and extends similar results obtained with this compound in other cancer types [45-47]. Importantly, the failure of PRIMA-1^{MET} to induce transactivation of p53 targets in mutp53 GBM cells (p21, Bax) suggests that it acted independently of restoration of wild-type conformation and DNA-binding ability of mutp53 in the studied cell lines.

In our study by showing increased sensitivity of MGMT low or negative GBM cells manifested in decreased cell number, proliferation and clonogenic capability, we reveal for the first time that MGMT is relevant for response of GBM cells to PRIMA-1^{MET}. This suggests that MGMT might protect GBM cells from the cytotoxicity of this compound. Moreover, we describe a novel finding that PRIMA-1^{MET} mechanism of action differs in MGMT low/ negative cells possessing wt or mutp53 (Figure 4.1). Our finding that in MGMT low / mutp53 GBM cells (established and GSCs) PRIMA-1^{MET} induced decreased levels of mutp53 protein are in agreement with results of Russo et al. showing that precursor analogue PRIMA-1 induced nucleolar redistribution of mutp53 with its subsequent degradation in breast cancer cells [48]. What this may suggest is that by downregulation of mutp53 PRIMA-1^{MET} nullifies its GOF activities and increases GBM cell susceptibility to apoptotic cell death. Whether and, if so, how PRIMA-1^{MET} facilitates the degradation of mutp53 in GBM cells requires additional investigation. Although PRIMA-1^{MET}-induced apoptosis (increase in cleaved PARP and G0/G1 cell population) in MGMT low / mutp53 GBM cells is in accordance with PRIMA-1^{MET} effects described in cells from other cancer types [49], we suggest novel mediators of this cell fate decision. Particularly, PRIMA-1^{MET}-induced increase in GADD45A levels in the absence of main p53 target, p21, appears to occur in

p53-independent manner. This is in accordance with studies showing the selective role of GADD45A in the G2/M checkpoint and its function as a tumor suppressor protein through pro-apoptotic and growth suppression activities [50], possibly supported by a mechanism involving GADD45-induced inhibition of the kinase activity of the cdc2/cyclin B1 complex [51]. Moreover, as decrease in mutp53 levels was shown to facilitate activation of several p53 targets, including GADD45A [52], PRIMA-1^{MET}-induced downregulation of mutp53 might contribute to upregulation of this protein in MGMT low GBM cells. Importantly, expression and pro-apoptotic activity of GADD45A can be regulated through the mitogen-activated protein kinase (MAPK) signaling pathway including c-Jun-NH2-kinase (JNK) [50], which is involved in PRIMA-1-induced apoptosis in mutp53 cells, as shown in malignant colon cell lines [53]. Activation of JNK pathway could be at least partially responsible for upregulation of GADD45A leading to apoptosis in MGMT low/ mutp53 GBM cells.

The observation of sustained phosphorylation of Erk1/2 kinases (p-Erk1/2) provides evidence of this being another novel mediator of PRIMA-1^{MET}-induced apoptosis in MGMT low/ mutp53 GBM cells and GSC lines. This is in accordance with increasing evidence of pro-apoptotic activity of sustained p-Erk1/2 in different cancer types [54, 55], in contrast to the well-known pro-survival effects of transient p-Erk1/2 activation. These intriguing results provide the basis for future work that will focus on the identification of the immediate downstream effects of p-Erk1/2 activation that lead to apoptosis.

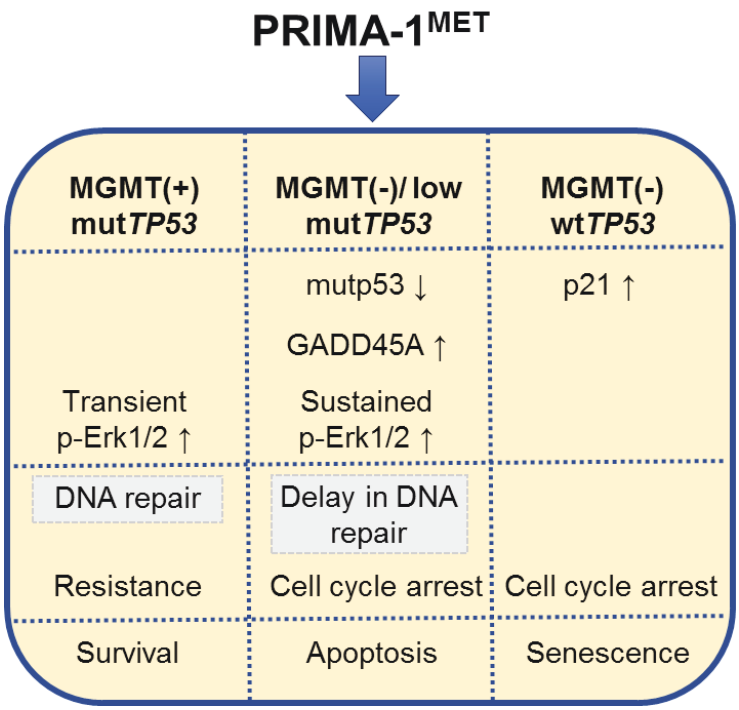

Figure 4.1. Potential mechanisms of action of PRIMA-1^{MET} in GBM cells with different MGMT levels and *TP53* status. The suggested PRIMA-1^{MET} effects that were not validated experimentally are highlighted in grey.

Our findings that PRIMA-1^{MET} demonstrates cytotoxic effects in wtp53 GBM cells, as previously mentioned, corroborates previous reports on PRIMA-1^{MET} effects irrespective of p53 status in other cancer types. Our study, however, shows for the first time that PRIMA-1^{MET} is able to trigger the development of senescent phenotype, rather than apoptosis, in MGMT negative / wt p53 GBM cells, as shown in U87MG cells. Strong basal levels of main p53 target, p21, in this cell line potentially heightened its sensitivity to PRIMA-1^{MET} inducing G1/M arrest and leading to senescence, which is in accordance with the known role of p21 in cell cycle regulation and senescence [56, 57]. Our observation of this differential cell fate decision in MGMT negative / wt p53 following exposure to PRIMA-1^{MET} is limited to one cell line and further studies will need to

investigate this phenomenon in additional cell lines and rule out whether PRIMA-1^{MET}-induced senescent phenotype is irreversible, as p53-intact senescent cells may maintain the ability to re-proliferate and escape senescence [58]. Our observation that PRIMA-1^{MET} induced increase in wtp53 accompanied by downregulation of MGMT levels in MGMT-positive GSCs (OPK111) corroborates, once again, the ability of wtp53 to down-modulate MGMT [1, 2], but also underlines the relevance of MGMT levels for response to PRIMA-1^{MET}.

The fascinating findings of this part of our study will hopefully serve as a guidance for future directions of the research on effects of PRIMA-1^{MET} in GBM. One aspect would be to overcome the limitations of this study by using a larger panel of GBM cell lines with different MGMT/ p53 profiles and modulating their MGMT levels (O6-BG, siRNA, exogenous expression, etc.) in order to corroborate our findings on increased sensitivity of MGMT low GBM cells to PRIMA-1^{MET} and differential cell fate based on their p53 status. Conducting the in-depth analysis of the molecular pathways altered by PRIMA-1^{MET} will aid in understanding the exact mechanisms of PRIMA-1^{MET}-induced apoptosis through GADD45A and sustained p-Erk1/2 and senescence through p21. Finally, anti-tumor effects and PRIMA-1^{MET} ability to efficiently cross the BBB will need to be tested in a GBM orthotopic model *in vivo*.

4.3 THE POTENCY OF PRIMA-1^{MET} AS A RADIOSENSITIZER IN GBM CELLS WITH DIFFERENT MGMT LEVELS AND *TP53* STATUS

Tumor resistance to RT, a key component of standard therapy in GBM patients, resulting from extremely complex intertumoral and intratumoral heterogeneity and multiple radioresistance mechanisms (intrinsic and extrinsic) is the major reason for treatment failure [59, 60]. Mutations

in *TP53*, reported in ~30% of primary GBM [61], may lead to aberrant response to IR due to either failure to activate p53 targets required for efficient DNA repair or apoptosis, dominant negative inhibition of residual wild-type p53 protein functions or GOF mutp53-induced activation of radioresistance mechanisms (increased DNA repair efficiency, prevention of apoptosis, altered gene expression and signaling pathways) [32, 33, 62]. In this part of the study (Chapter 3) we assessed for the first time the potential of p53-targeting by PRIMA-1^{MET} as an approach to sensitize GBM cells to IR. We sought to explore whether the restoration of p53 functions induced by pre-treatment of GBM cells with PRIMA-1^{MET} would facilitate activation of p53-dependent pro-apoptotic cell signalling following exposure to IR, ultimately leading to cell death.

Our findings that pre-treatment with PRIMA-1^{MET} prior to exposure to IR significantly enhanced inhibition of cell number, proliferation and clonogenic survival in MGMT low / negative GBM cells, compared to PRIMA-1^{MET} or IR alone, is in agreement with our results for PRIMA-1^{MET} used as a single agent. Our data also extend the previous observations that MGMT silencing is necessary to achieve radiosensitizing effects in GBM xenografts *in vivo* [16] and suggestions that MGMT protects cells from DNA damage caused by IR [10]. Similar to our results with PRIMA-1^{MET} alone, its combination with IR caused cytotoxicity in MGMT low / negative GBM cells irrespective of their p53 status. This corroborates the observation of PRIMA-1^{MET} ability to radiosensitize prostate cancer cells in a mutp53-independent manner [46].

In accordance with our findings with PRIMA-1^{MET} used as a single agent, the cell fate following exposure to the combination of PRIMA-1^{MET} and IR differed in MGMT low / negative GBM cells possessing wt or mutp53 (Figure 4.2). Specifically, the combination-induced upregulation of cleaved forms of PARP and caspase-3 indicates that MGMT low / mutp53 GBM cells undergo cell death through apoptosis, which agrees with widely reported pro-apoptotic

activity of PRIMA-1^{MET} [49] and our own data showing that apoptosis is induced selectively in MGMT low / mutp53 GBM cells by this compound. The observed combination treatment-induced decrease in mutp53 protein levels extends our findings with PRIMA-1^{MET} alone and suggests that inhibition of GOF activities of p53 mutants is a relevant factor for PRIMA-1^{MET} cytotoxicity and sensitization of GBM cells to IR. The failure of PRIMA-1^{MET} combined with IR to induce wtp53 target, p21, in MGMT low / mutp53 GBM cells, similar to our results with PRIMA-1^{MET} alone, suggests that apoptosis is induced independent of restoration of normal functions to mutp53. In our study we report for the first time that PRIMA-1^{MET} alone and combined with IR is able to induce increased ɣ-H2AX levels and foci formation, a hallmark of DNA DSBs, in MGMT low / mutp53 GBM cells. To our knowledge, the PRIMA-1^{MET} capability to induce DNA damage and DNA DSBs, in particular, has not been previously reported. This intriguing finding agrees with suggested reduced capability of mutp53 glioma cells to efficiently repair DNA DSBs [62]. Moreover, this corroborates the notion that MGMT activity ensures a protective role in response to mutagenic effects of chemo- and radiotherapy [10], which is in accordance with reports showing correlation between MGMT silencing through promoter methylation and improved response to radiotherapy in GBM patients [63] and in GSCs *in vitro* [64]. The relevance of MGMT for response to IR is theoretically not limited to GBM as MGMT knockdown enhanced radiosensitivity and was associated with increased ɣ-H2AX and cleaved caspase-3 in breast cancer cells [65]. At the current stage of investigation, it is not possible to conclude whether DNA damage manifesting by phosphorylation of histone H2AX (ɣ-H2AX) is caused by PRIMA-1^{MET} directly or through regulation of other cellular factors. Importantly, PRIMA-1^{MET} is able to bind substrates other than p53, such as glutathione and thioredoxin reductase 1, in cells irrespective of their p53 status [66, 67]. These two proteins are important for regulation of cellular redox homeostasis, so

by blocking their activity PRIMA-1^{MET} affects cellular redox status leading to production of ROS and potentially causing oxidative DNA damage [66]. Understanding whether PRIMA-1^{MET} promotes an oxidative environment leading to DNA damage in GBM cells is crucial for elucidation of its mechanism of action in this type of brain tumors. This novel DNA damaging capability of PRIMA-1^{MET} is especially important in the context of extreme radioresistance of GBM tumors, as its combination with IR could potentially induce unbearable levels of DNA damage in GBM cells and lead to massive apoptosis.

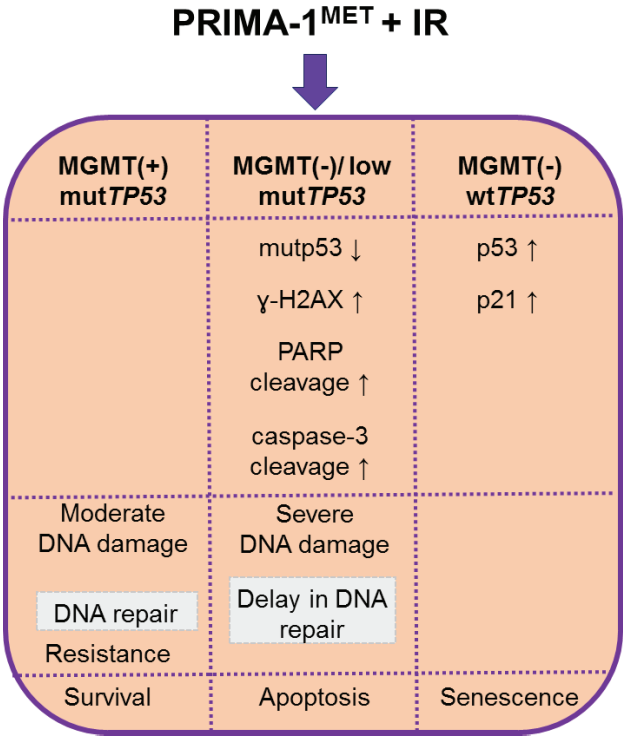

Figure 4.2. Potential mechanisms of action of PRIMA-1^{MET} combined with IR in GBM cells with different MGMT levels and *TP53* status. The suggested effects that were not validated experimentally are highlighted in grey.

The alternative cell fate decision – development of senescent phenotype following exposure to PRIMA-1^{MET} and IR in MGMT low / wtp53 GBM cells is in accordance with our results showing the effects of PRIMA-1^{MET} as a single agent [68]. The finding that combined treatment induced senescence in both MGMT low / wtp53 cell lines (U87MG and A172) is in accordance with their previously reported IR-induced senescence [69, 70]. The observation that only U87MG undergoes senescence in response to PRIMA-1^{MET} alone suggests that there are other protective factors, in addition to MGMT, involved in resistance of A172 cells to PRIMA-1^{MET} cytotoxicity. Early activation of p53 with subsequent upregulation of p21 in MGMT low / wtp53 cells suggests that senescence occurs through p53/$p21^{waf1}$ signaling, rather than $p16^{INK4a}$/ Rb pathway [71], which is corroborated by the reports of $p16^{INK4a}$ deletion in these cell lines [72, 73].

Alongside these exciting findings it is also necessary to state that this part of our study suffers some limitations that need to be overcome in future investigations. Similarly to the previous section of the study, our data on effects of PRIMA-1^{MET} combined with IR and the role of MGMT activity and *TP53* status would need to be validated in a larger panel of GBM cell lines. Our intriguing novel finding that PRIMA-1^{MET} induces DNA damage in MGMT low / mutp53 GBM cells lay the foundation for future work to gain better insights into extent of DNA damage caused by this compound by utilizing specific assays and assessing the alterations in additional proteins involved in DNA damage response (Rad51, XRCC4, etc.) and understand the mechanism of this process with emphasis on the potential role of ROS. Importantly, as TMZ is a key component of standard treatment for GBM patients, it would be crucial to test the effects of PRIMA-1^{MET}+TMZ combination with and without IR, especially with regards to the reports of synergy between PRIMA-1^{MET} and a number of DNA-damaging compounds (e.g. cisplatin, doxorubicin,

gemcitabine) in other cancer types [44, 74, 75]. Finally, safety, pharmacokinetic, and antitumor activity of PRIMA-1^{MET} combined with IR and TMZ will need to be tested *in vivo*.

In summary, our study is the first to suggest the reciprocal relationship between MGMT and p53 in GBM and potentially other cancer types. Our findings also propose that the effects of PRIMA-1^{MET} are independent of restoring wtp53 functions to mutp53 in the studied GBM cells, while the mechanism of cytotoxicity and cell fate is different in MGMT low cells possessing wt or mutp53. Our results show that PRIMA-1^{MET} is capable of causing DNA damage and, thus, facilitates the cytotoxicity of IR MGMT low / mutp53. This suggests that the levels of MGMT and p53 status are relevant for this activity due to potential MGMT/ mutp53 relationship and the role of these proteins in DNA damage response. Despite the aforementioned limitations of our study, we provided new insights into the complex nature of regulation of MGMT and p53 and their role in response to targeted treatment. While working on this project, we kept in mind that GBM represents a highly complex, heterogeneous and multifactorial set of diseases not allowing simple answers or straightforward approaches for characterization of mechanisms of tumor cell functioning and accurate prediction of response to specific treatments. Still, we hope that future studies will provide a better understanding of MGMT/ p53 crosstalk in patients with GBM and other types of cancer, potentially allowing its use to predict efficacy of PRIMA-1^{MET} as a novel personalized therapeutic strategy to improve the standard of care for patients suffering from this devastating disease.

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APPENDICES

Table A1. Normalized mRNA expression data (z-score values) of MGMT and p53 and *TP53* status in Cancer Cell Line Encyclopedia (CCLE) human cancer cell lines dataset

Cell line	Primary site	Histology	MGMT mRNA z score	TP53 mRNA z score	TP53 Mutant	Mutation Type
697	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.13	1.31	no	
5637	urinary_tract	carcinoma	-1.89	0.43	yes	Missense Mutation
1321n1	central_nervous_system	glioma	0.31	0.58	no	
143b	bone	osteosarcoma	-2.35	0.66	no	
22rv1	prostate	carcinoma	-2.35	-0.77	yes	Missense Mutation
23132/87	stomach	carcinoma	0.78	0.77	no	
42-mg-ba	central_nervous_system	glioma	-1.45	-0.11	yes	Missense Mutation
639-v	urinary_tract	carcinoma	-1.00	0.90	yes	Missense Mutation
647-v	urinary_tract	carcinoma	-2.35	1.08	yes	Nonsense Mutation
769-p	kidney	carcinoma	1.03	0.25	no	
786-o	kidney	carcinoma	0.59	-0.82	yes	Missense Mutation
8305c	thyroid	carcinoma	-0.78	0.92	yes	Missense Mutation
8505c	thyroid	carcinoma	-0.55	0.88	yes	Missense Mutation

8-mg-ba	central_nervous_system	glioma	-1.49	0.76	yes	Missense Mutation
a101d	skin	malignant_melanoma	-2.30	0.04	no	
a172	central_nervous_system	glioma	-2.00	0.90	no	
a-204	soft_tissue	rhabdomyosarcoma	0.80	0.57	no	
a-253	salivary_gland	carcinoma	-2.35	-1.82	yes	Frame Shift Del
a2780	ovary	carcinoma	0.19	0.43	no	
a3/kaw	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.04	-0.43	no	
a-375	skin	malignant_melanoma	0.42	0.40	no	
a4/fuk	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.35	0.32	yes	Missense Mutation
a-498	kidney	carcinoma	0.51	0.79	no	
a549	lung	carcinoma	-0.29	0.34	no	
a-673	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	1.05	-1.05	no	
a-704	kidney	carcinoma	0.60	-0.38	no	
abc-1	lung	carcinoma	0.49	0.84	yes	Missense Mutation
acc-meso-1	pleura	mesothelioma	0.44	-2.05	no	
achn	kidney	carcinoma	0.75	0.36	no	
ags	stomach	carcinoma	0.89	-0.03	no	
alexander cells	liver	carcinoma	-0.06	-0.19	yes	Missense Mutation
am-38	central_nervous_system	glioma	-2.35	-0.42	no	
aml-193	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.65	-1.95	yes	Splice Site SNP

amo-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.35	0.77	no	
an3 ca	endometrium	carcinoma	0.76	0.24	yes	Frame Shift Del
aspc-1	pancreas	carcinoma	0.89	-1.59	yes	Frame Shift Del
au565	breast	carcinoma	0.35	0.26	yes	Missense Mutation
az-521	stomach	carcinoma	0.08	0.65	no	
bc-3c	urinary_tract	carcinoma	0.32	1.04	no	
bcp-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.17	0.50	yes	Missense Mutation
b-cpap	thyroid	carcinoma	-2.35	0.95	yes	Missense Mutation
bdcm	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.53	0.78	no	
becker	central_nervous_system	glioma	-1.29	0.73	yes	Splice Site SNP
ben	lung	carcinoma	-0.91	-0.34	yes	Missense Mutation
bftc-905	urinary_tract	carcinoma	-0.86	-1.19	yes	Splice Site SNP
bftc-909	kidney	carcinoma	0.16	0.24	yes	Nonsense Mutation
bht-101	thyroid	carcinoma	-2.17	0.83	no	
bhy	upper_aerodigestive_tract	carcinoma	0.12	-1.45	no	
bicr 16	upper_aerodigestive_tract	carcinoma	0.76	-1.06	yes	Nonsense Mutation
bicr 18	upper_aerodigestive_tract	carcinoma	0.91	-1.12	no	

bicr 22	upper_aerodigestive_tract	carcinoma	-2.35	-1.26	no	
bicr 31	upper_aerodigestive_tract	carcinoma	-0.02	0.84	yes	In Frame Del
bicr 56	upper_aerodigestive_tract	carcinoma	-0.59	0.25	yes	Splice Site SNP
bicr 6	upper_aerodigestive_tract	carcinoma	-0.20	-1.19	yes	Nonsense Mutation
bl-41	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.02	-0.35	yes	Missense Mutation
bl-70	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.11	1.20	yes	Missense Mutation
bt-20	breast	carcinoma	0.34	1.02	yes	Missense Mutation
bt-474	breast	carcinoma	0.12	0.84	yes	Missense Mutation
bt-483	breast	carcinoma	0.27	0.19	yes	Missense Mutation
bt-549	breast	carcinoma	0.53	1.56	no	
bv-173	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.64	0.76	no	
bxpc-3	pancreas	carcinoma	0.96	0.99	yes	Missense Mutation
c2bbe1	large_intestine	carcinoma	1.12	-1.54	yes	Nonsense Mutation
c32	skin	malignant_melanoma	-2.35	-0.12	no	
c3a	liver	carcinoma	0.62	0.29	no	
ca46	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.50	0.98	yes	Missense Mutation
cado-es1	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	0.82	0.55	no	

caki-1	kidney	carcinoma	1.43	-0.04	no	
caki-2	kidney	carcinoma	1.12	0.38	no	
cal 27	upper_aerodigestive_tract	carcinoma	0.40	0.90	yes	Missense Mutation
cal-120	breast	carcinoma	0.26	-0.70	yes	Splice Site SNP
cal-12t	lung	carcinoma	0.13	0.84	yes	Missense Mutation
cal-148	breast	carcinoma	0.96	1.19	yes	Missense Mutation
cal-29	urinary_tract	carcinoma	0.80	0.30	yes	Missense Mutation
cal-51	breast	carcinoma	0.48	0.73	no	
cal-54	kidney	carcinoma	0.66	0.97	no	
cal-62	thyroid	carcinoma	0.80	0.82	yes	Missense Mutation
cal-78	bone	chondrosarcoma	-0.15	0.04	yes	In Frame Del
cal-85-1	breast	carcinoma	0.61	1.15	yes	Missense Mutation
calu-1	lung	carcinoma	0.41	-2.31	no	
calu-3	lung	carcinoma	-0.24	0.55	yes	Missense Mutation
calu-6	lung	carcinoma	0.60	-1.19	yes	Nonsense Mutation
cama-1	breast	carcinoma	0.75	0.00	yes	Missense Mutation
caov-3	ovary	carcinoma	0.55	-1.97	yes	Nonsense Mutation
caov-4	ovary	carcinoma	0.49	0.46	yes	Missense Mutation
capan-1	pancreas	carcinoma	0.85	0.61	yes	Missense Mutation

capan-2	pancreas	carcinoma	1.06	-1.08	no	
cas-1	central_nervous_system	glioma	-1.05	0.84	yes	Missense Mutation
ccf-sttg1	central_nervous_system	glioma	-2.35	-0.14	no	
cck-81	large_intestine	carcinoma	0.86	0.99	yes	Missense Mutation
cfpac-1	pancreas	carcinoma	0.19	-0.28	yes	Missense Mutation
cgth-w-1	thyroid	carcinoma	0.32	0.60	yes	Missense Mutation
chago-k-1	lung	carcinoma	0.20	0.43	yes	Missense Mutation
chl-1	skin	malignant_melanoma	0.26	0.29	yes	Missense Mutation
chp-126	autonomic_ganglia	neuroblastoma	0.23	0.28	no	
chp-212	autonomic_ganglia	neuroblastoma	-1.27	0.62	no	
ci-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.46	1.45	yes	Missense Mutation
cjm	skin	malignant_melanoma	-1.24	0.13	yes	Missense Mutation
cl-11	large_intestine	carcinoma	0.35	-0.01	yes	Missense Mutation
cl-14	large_intestine	carcinoma	-0.04	0.59	yes	Missense Mutation
cl-34	large_intestine	carcinoma	0.83	1.33	yes	Missense Mutation
cl-40	large_intestine	carcinoma	-0.04	0.92	yes	Missense Mutation
cmk	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.50	-2.84	yes	Missense Mutation

cmk-11-5	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.24	-2.21	yes	Missense Mutation
cml-t1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.81	0.81	yes	Missense Mutation
colo 205	large_intestine	carcinoma	0.94	-0.05	no	
colo 668	lung	carcinoma	0.11	0.02	yes	Missense Mutation
colo 741	skin	malignant_melanoma	-0.46	-1.45	yes	Frame Shift Ins
colo 792	skin	malignant_melanoma	-2.17	0.03	no	
colo 829	skin	malignant_melanoma	-0.06	0.27	no	
colo-320	large_intestine	carcinoma	-2.35	0.63	yes	Missense Mutation
colo-678	large_intestine	carcinoma	-0.07	0.31	no	
colo-679	skin	malignant_melanoma	-0.64	-0.34	no	
colo-680n	oesophagus	carcinoma	0.48	1.20	yes	Missense Mutation
colo-704	ovary	carcinoma	-0.02	-2.71	no	
colo-783	skin	malignant_melanoma	0.88	0.41	yes	Missense Mutation
colo-800	skin	malignant_melanoma	-2.35	0.63	no	
colo-818	skin	malignant_melanoma	-2.35	0.28	yes	Missense Mutation
colo-849	skin	malignant_melanoma	0.27	0.76	no	
cor-l105	lung	carcinoma	0.50	-0.26	no	
cor-l23	lung	carcinoma	0.31	1.14	no	
cor-l24	lung	carcinoma	0.89	-0.36	yes	Missense Mutation
cor-l311	lung	carcinoma	0.87	-0.22	no	
cor-l47	lung	carcinoma	-2.25	-1.56	yes	Nonsense Mutation

cor-l88	lung	carcinoma	-1.94	-0.03	yes	Missense Mutation
cor-l95	lung	carcinoma	-1.64	-0.20	yes	Missense Mutation
cov318	ovary	carcinoma	0.60	0.17	yes	Missense Mutation
cov362	ovary	carcinoma	0.88	0.82	yes	Missense Mutation
cov434	ovary	sex_cord-stromal_tumour	0.48	0.30	no	
cov504	ovary	carcinoma	0.42	-1.12	yes	Frame Shift Del
cov644	ovary	carcinoma	0.74	-3.65	no	
cpc-n	lung	carcinoma	0.48	0.49	yes	Missense Mutation
cw-2	large_intestine	carcinoma	0.09	1.32	no	
d283 med	central_nervous_system	primitive_neuroectodermal_tumour-medulloblastoma	0.45	0.35	no	
d341 med	central_nervous_system	primitive_neuroectodermal_tumour-medulloblastoma	0.35	-0.32	no	
dan-g	pancreas	carcinoma	0.33	-1.30	no	
daoy	central_nervous_system	primitive_neuroectodermal_tumour-medulloblastoma	-0.36	1.16	yes	Missense Mutation
daudi	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.69	0.70	yes	Missense Mutation
db	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.13	0.86	yes	Nonsense Mutation
dbtrg-05mg	central_nervous_system	glioma	-1.18	0.23	no	
del	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.35	0.83	yes	Missense Mutation
detroit 562	upper_aerodigestive_tract	carcinoma	0.53	1.19	yes	Missense Mutation

dk-mg	central_nervous_system	glioma	-2.35	0.01	no	
dld-1	large_intestine	carcinoma	0.62	-0.13	no	
dms 114	lung	carcinoma	1.06	-1.21	yes	Nonsense Mutation
dms 153	lung	carcinoma	0.31	-0.36	yes	Missense Mutation
dms 273	lung	carcinoma	0.76	0.45	yes	Missense Mutation
dms 454	lung	carcinoma	-2.35	-0.58	yes	Missense Mutation
dms 53	lung	carcinoma	-1.41	-0.03	yes	Missense Mutation
dms 79	lung	carcinoma	-0.07	-0.25	yes	Frame Shift Del
dnd-41	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.02	0.96	yes	Missense Mutation
dohh-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.19	1.24	no	
du 145	prostate	carcinoma	0.62	0.63	yes	Missense Mutation
du4475	breast	carcinoma	-0.04	-0.37	no	
dv-90	lung	carcinoma	0.59	-0.39	no	
eb1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.49	1.09	no	
eb2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.31	1.21	yes	Missense Mutation
ebc-1	lung	carcinoma	0.31	-1.91	yes	Nonsense Mutation
ecc10	stomach	carcinoma	0.41	-1.08	no	
ecc12	stomach	carcinoma	0.55	-0.45	yes	Missense Mutation

ec-gi-10	oesophagus	carcinoma	-1.69	0.99	yes	Missense Mutation
efe-184	endometrium	carcinoma	0.62	0.82	yes	Missense Mutation
efm-19	breast	carcinoma	-0.43	-0.06	yes	Missense Mutation
efm-192a	breast	carcinoma	0.74	-2.23	yes	Frame Shift Ins
efo-21	ovary	carcinoma	0.52	1.47	no	
efo-27	ovary	carcinoma	1.09	0.77	yes	Missense Mutation
eheb	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.86	0.56	no	
ejm	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.06	0.15	yes	Missense Mutation
em-2	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.21	0.58	yes	Missense Mutation
en	endometrium	carcinoma	0.92	0.11	no	
eol-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.88	1.08	no	
eplc-272h	lung	carcinoma	0.22	-1.60	no	
es-2	ovary	carcinoma	-2.30	-0.12	yes	Missense Mutation
ess-1	endometrium	carcinoma	0.79	-0.96	yes	Nonsense Mutation
f-36p	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-1.30	0.65	yes	Splice Site SNP
fadu	upper_aerodigestive_tract	carcinoma	0.70	0.15	yes	Missense Mutation
ftc-133	thyroid	carcinoma	0.64	0.88	no	
fu97	stomach	carcinoma	1.25	-0.27	yes	Missense Mutation

fu-ov-1	ovary	carcinoma	0.92	1.02	yes	Missense Mutation
g-292, clone a141b1	bone	osteosarcoma	0.87	-2.81	no	
g-361	skin	malignant_melanoma	0.38	-0.70	no	
g-401	soft_tissue	rhabdoid_tumour	0.89	0.28	no	
g-402	soft_tissue	rhabdoid_tumour	0.31	0.50	no	
ga-10	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.76	0.78	no	
gamg	central_nervous_system	glioma	-1.24	-0.14	yes	Missense Mutation
gciy	stomach	carcinoma	0.67	0.01	yes	Missense Mutation
gct	soft_tissue	malignant_fibrous_histiocytoma-pleomorphic_sarcoma	-1.01	-0.05	yes	Nonsense Mutation
gdm-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.00	0.74	no	
gi-1	central_nervous_system	glioma	-2.30	0.60	yes	Missense Mutation
gms-10	central_nervous_system	glioma	-2.35	0.65	yes	Missense Mutation
gos-3	central_nervous_system	glioma	-1.36	0.50	no	
gp2d	large_intestine	carcinoma	1.34	0.69	no	
granta-519	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.00	1.37	no	
gss	stomach	carcinoma	-2.30	0.21	yes	Missense Mutation
gsu	stomach	carcinoma	-0.18	0.85	yes	Missense Mutation
h4	central_nervous_system	glioma	-0.10	0.44	no	

hara	lung	carcinoma	0.99	0.55	yes	Missense Mutation
hcc1143	breast	carcinoma	0.44	1.14	yes	Missense Mutation
hcc-1171	lung	carcinoma	0.36	0.27	yes	Missense Mutation
hcc1187	breast	carcinoma	0.45	1.10	no	
hcc-1195	lung	carcinoma	0.87	0.67	yes	Splice Site Del
hcc1395	breast	carcinoma	0.26	1.18	yes	Missense Mutation
hcc1419	breast	carcinoma	0.41	-0.22	yes	Missense Mutation
hcc1428	breast	carcinoma	0.22	0.79	no	
hcc-15	lung	carcinoma	0.75	0.13	yes	Missense Mutation
hcc1500	breast	carcinoma	0.48	0.31	no	
hcc1569	breast	carcinoma	1.08	-1.00	yes	Nonsense Mutation
hcc1599	breast	carcinoma	0.31	-1.02	yes	Splice Site SNP
hcc1806	breast	carcinoma	0.83	-0.47	no	
hcc1937	breast	carcinoma	1.38	-1.23	no	
hcc1954	breast	carcinoma	-0.25	0.53	yes	Missense Mutation
hcc202	breast	carcinoma	0.50	-2.00	yes	Frame Shift Del
hcc2157	breast	carcinoma	0.84	0.41	yes	Missense Mutation
hcc2218	breast	carcinoma	0.63	0.18	no	
hcc-2279	lung	carcinoma	-0.11	0.91	yes	Missense Mutation

hcc2935	lung	carcinoma	0.60	1.22	yes	Missense Mutation
hcc-33	lung	carcinoma	0.19	0.56	yes	Missense Mutation
hcc-366	lung	carcinoma	-0.87	0.07	yes	Missense Mutation
hcc38	breast	carcinoma	0.62	0.04	yes	Missense Mutation
hcc4006	lung	carcinoma	0.12	1.19	no	
hcc-44	lung	carcinoma	0.09	-2.33	yes	Missense Mutation
hcc-56	large_intestine	carcinoma	0.90	0.30	yes	Missense Mutation
hcc70	breast	carcinoma	0.66	0.57	yes	Missense Mutation
hcc-78	lung	carcinoma	0.72	0.09	yes	Missense Mutation
hcc827	lung	carcinoma	0.26	0.58	yes	In Frame Del
hcc-95	lung	carcinoma	0.87	-0.36	no	
hct 116	large_intestine	carcinoma	0.12	0.13	no	
hct-15	large_intestine	carcinoma	0.56	0.08	yes	Missense Mutation
hdlm-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.21	-3.65	no	
hd-my-z	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.14	-1.80	yes	Frame Shift Del
hdq-p1	breast	carcinoma	0.35	-0.73	no	
hec-108	endometrium	carcinoma	0.73	0.42	yes	Missense Mutation
hec-151	endometrium	carcinoma	1.01	0.35	no	
hec-1-a	endometrium	carcinoma	0.99	0.83	yes	Missense Mutation

hec-1-b	endometrium	carcinoma	0.78	0.72	yes	Missense Mutation
hec-251	endometrium	carcinoma	0.35	-0.24	yes	Nonsense Mutation
hec-265	endometrium	carcinoma	0.92	-0.07	no	
hec-50b	endometrium	carcinoma	0.37	-1.56	no	
hec-59	endometrium	carcinoma	0.57	0.15	no	
hec-6	endometrium	carcinoma	0.64	-0.34	no	
hel	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.12	0.51	yes	Missense Mutation
hel 92.1.7	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.03	0.00	yes	Missense Mutation
hep 3b2.1-7	liver	carcinoma	0.11	-3.65	no	
hep g2	liver	carcinoma	0.60	0.10	no	
hey-a8	ovary	carcinoma	-1.52	0.50	no	
hgc-27	stomach	carcinoma	0.59	-1.71	yes	Frame Shift Ins
hh	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.19	-1.54	yes	Splice Site SNP
hle	liver	carcinoma	0.20	-0.36	no	
hlf	liver	carcinoma	0.50	0.26	yes	Missense Mutation
hlf-a	lung	carcinoma	0.53	-0.21	no	
hmcb	skin	malignant_melanoma	-0.63	0.32	yes	Missense Mutation
hos	bone	osteosarcoma	-0.62	0.98	yes	Missense Mutation
hpac	pancreas	carcinoma	0.46	-1.85	no	
hpaf-ii	pancreas	carcinoma	1.08	0.05	yes	Missense Mutation
hpb-all	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.53	-1.44	no	

hs 172.t	urinary_tract	carcinoma	1.01	-0.28	no	
hs 229.t	lung	carcinoma	-0.04	-0.20	no	
hs 274.t	breast	carcinoma	0.12	0.19	no	
hs 281.t	breast	carcinoma	-0.65	0.12	no	
hs 294t	skin	malignant_melanoma	-0.88	0.82	no	
hs 343.t	breast	carcinoma	0.40	0.19	no	
hs 578t	breast	carcinoma	-2.17	0.41	yes	Missense Mutation
hs 600.t	skin	malignant_melanoma	0.34	0.03	no	
hs 604.t	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.40	0.35	no	
hs 606.t	breast	carcinoma	0.32	0.22	no	
hs 611.t	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.05	0.50	no	
hs 616.t	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.59	0.22	no	
hs 618.t	lung	carcinoma	-0.10	-0.54	no	
hs 675.t	large_intestine	carcinoma	0.74	-0.23	no	
hs 683	central_nervous_system	glioma	-0.48	-0.05	yes	Missense Mutation
hs 688(a).t	skin	malignant_melanoma	0.75	-0.22	no	
hs 695t	skin	malignant_melanoma	0.72	0.76	no	
hs 698.t	large_intestine	carcinoma	0.40	0.27	no	
hs 706.t	bone	giant_cell_tumour	0.15	-0.12	no	
hs 729	soft_tissue	rhabdomyosarcoma	0.08	-0.02	yes	Missense Mutation
hs 737.t	bone	other	0.53	0.19	no	
hs 739.t	breast	carcinoma	0.81	0.18	no	
hs 742.t	breast	carcinoma	0.61	-0.15	no	
hs 746t	stomach	carcinoma	-1.57	-1.57	yes	Nonsense Mutation

hs 751.t	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.22	0.38	no	
hs 766t	pancreas	carcinoma	0.91	0.60	no	
hs 819.t	bone	chondrosarcoma	0.42	-0.20	no	
hs 821.t	bone	giant_cell_tumour	-0.03	0.87	no	
hs 822.t	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	0.14	0.05	no	
hs 839.t	skin	malignant_melanoma	0.68	-0.23	yes	Missense Mutation
hs 840.t	upper_aerodigestive_tract	other	0.47	-0.04	no	
hs 852.t	skin	malignant_melanoma	-0.47	-0.17	no	
hs 863.t	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	0.21	0.00	no	
hs 870.t	bone	osteosarcoma	0.63	-0.50	no	
hs 888.t	bone	osteosarcoma	0.44	0.24	no	
hs 895.t	skin	malignant_melanoma	0.63	-0.75	no	
hs 934.t	skin	malignant_melanoma	0.57	0.33	no	
hs 936.t	skin	malignant_melanoma	0.41	-0.73	no	
hs 939.t	skin	malignant_melanoma	-1.06	-0.39	no	
hs 940.t	skin	malignant_melanoma	0.42	-0.06	no	
hs 944.t	skin	malignant_melanoma	-0.24	-0.24	no	
hsc-2	upper_aerodigestive_tract	carcinoma	0.61	-0.93	yes	Splice Site SNP
hsc-3	upper_aerodigestive_tract	carcinoma	0.25	-1.68	yes	Frame Shift Ins
hsc-4	upper_aerodigestive_tract	carcinoma	0.93	0.77	yes	Missense Mutation

ht	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.28	1.02	yes	Missense Mutation
ht-1080	soft_tissue	fibrosarcoma	-0.19	0.03	no	
ht115	large_intestine	carcinoma	0.62	-1.64	yes	Nonsense Mutation
ht-1197	urinary_tract	carcinoma	-0.73	0.67	no	
ht-1376	urinary_tract	carcinoma	0.58	0.84	yes	Missense Mutation
ht-144	skin	malignant_melanoma	0.00	0.28	no	
ht-29	large_intestine	carcinoma	0.76	1.03	no	
ht55	large_intestine	carcinoma	0.84	0.93	no	
hucct1	biliary_tract	carcinoma	0.16	0.22	yes	Missense Mutation
hug1-n	stomach	carcinoma	-2.06	0.57	yes	Missense Mutation
huh-1	liver	carcinoma	1.01	0.12	no	
huh28	biliary_tract	carcinoma	-0.13	0.26	yes	Missense Mutation
huh-6	liver	other	0.85	0.26	yes	Missense Mutation
huh-7	liver	carcinoma	0.11	0.69	yes	Missense Mutation
huns1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.11	0.57	no	
hup-t3	pancreas	carcinoma	0.66	-0.17	yes	Missense Mutation
hup-t4	pancreas	carcinoma	0.83	0.93	yes	Missense Mutation
hut 102	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.03	0.24	no	
hut 78	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.67	-1.50	yes	Nonsense Mutation

hutu 80	small_intestine	carcinoma	0.78	0.40	no	
ia-lm	lung	carcinoma	0.56	-1.25	yes	Nonsense Mutation
igr-1	skin	malignant_melanoma	0.06	-0.96	no	
igr-37	skin	malignant_melanoma	0.20	-2.36	yes	Frame Shift Del
igr-39	skin	malignant_melanoma	0.50	-1.99	yes	Frame Shift Del
igrov1	ovary	carcinoma	0.15	0.16	yes	Missense Mutation
im95	stomach	carcinoma	0.74	0.76	no	
imr-32	autonomic_ganglia	neuroblastoma	0.24	0.56	no	
ipc-298	skin	malignant_melanoma	0.62	-1.81	yes	Nonsense Mutation
ishikawa (heraklio) 02 er-	endometrium	carcinoma	0.98	-0.24	yes	Missense Mutation
ist-mes1	pleura	mesothelioma	-0.06	0.66	no	
ist-mes2	pleura	mesothelioma	0.89	0.13	yes	Missense Mutation
j82	urinary_tract	carcinoma	0.67	-0.70	yes	Missense Mutation
jeko-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.17	-0.89	no	
jhh-1	liver	carcinoma	1.21	0.25	no	
jhh-2	liver	carcinoma	0.46	-1.03	no	
jhh-4	liver	carcinoma	-2.35	0.32	yes	Missense Mutation
jhh-5	liver	carcinoma	0.77	0.32	yes	In Frame Del
jhh-6	liver	carcinoma	0.54	-0.14	no	
jhh-7	liver	carcinoma	0.87	-0.34	yes	Missense Mutation
jhoc-5	ovary	carcinoma	-2.35	0.19	no	

jhom-1	ovary	carcinoma	-0.86	-0.55	yes	Nonsense Mutation
jhom-2b	ovary	carcinoma	0.40	0.56	yes	Missense Mutation
jhos-2	ovary	carcinoma	0.93	0.08	yes	Splice Site Del
jhos-4	ovary	carcinoma	1.31	0.22	yes	Missense Mutation
jhuem-1	endometrium	carcinoma	0.97	0.53	no	
jhuem-2	endometrium	carcinoma	0.62	0.77	no	
jhuem-3	endometrium	carcinoma	0.53	-1.44	no	
jimt-1	breast	carcinoma	0.82	1.52	yes	Missense Mutation
jk-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.14	0.77	no	
jl-1	pleura	mesothelioma	-0.25	1.39	no	
jm1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.14	1.23	no	
jmsu-1	urinary_tract	carcinoma	0.72	0.70	yes	Missense Mutation
jurkat	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.01	-0.46	no	
jurl-mk1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.26	-1.00	no	
jvm-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.21	0.53	no	
jvm-3	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.07	1.31	no	
k029ax	skin	malignant_melanoma	0.29	-0.13	no	
k-562	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-1.10	-1.42	yes	Frame Shift Ins

kals-1	central_nervous_system	glioma	0.57	0.44	yes	Missense Mutation
karpas-299	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.53	0.29	yes	Missense Mutation
karpas-620	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.18	1.25	yes	Missense Mutation
kasumi-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.90	0.30	yes	Missense Mutation
kasumi-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.13	0.83	yes	Missense Mutation
kasumi-6	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-1.08	0.52	yes	Missense Mutation
kato iii	stomach	carcinoma	-0.14	-3.65	no	
kci-moh1	pancreas	carcinoma	0.36	-1.35	no	
kcl-22	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.44	-0.82	yes	Frame Shift Del
ke-37	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.42	-0.16	no	
ke-39	stomach	carcinoma	0.74	0.29	yes	Missense Mutation
ke-97	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.27	0.41	no	
kelly	autonomic_ganglia	neuroblastoma	0.36	-0.09	yes	Missense Mutation
kg-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.59	-1.33	yes	Splice Site SNP
kg-1-c	central_nervous_system	glioma	0.54	0.41	no	
khm-1b	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.94	-1.41	no	
ki-jk	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.20	-0.14	no	

kle	endometrium	carcinoma	1.04	0.47	yes	Missense Mutation
km12	large_intestine	carcinoma	-1.97	0.30	yes	Missense Mutation
kmbc-2	urinary_tract	carcinoma	0.30	-1.15	yes	Missense Mutation
km-h2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.79	0.81	no	
kmm-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.64	-1.05	yes	Missense Mutation
kmrc-1	kidney	carcinoma	0.61	-0.25	no	
kmrc-2	kidney	carcinoma	0.49	0.82	no	
kmrc-20	kidney	carcinoma	0.67	-1.83	yes	Splice Site SNP
kmrc-3	kidney	carcinoma	0.64	-0.10	no	
kms-11	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.69	-3.45	no	
kms-12-bm	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.84	0.61	no	
kms-18	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.09	0.79	no	
kms-20	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.49	-1.15	yes	Nonsense Mutation
kms-21bm	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.20	-0.24	no	
kms-26	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.78	0.49	yes	Missense Mutation
kms-27	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.15	0.42	no	
kms-28bm	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.54	0.16	no	

kms-34	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.43	-1.50	yes	Nonsense Mutation
kns-42	central_nervous_system	glioma	-2.35	-0.45	yes	Nonsense Mutation
kns-60	central_nervous_system	glioma	0.00	0.88	yes	Missense Mutation
kns-62	lung	carcinoma	0.97	0.74	yes	Missense Mutation
kns-81	central_nervous_system	glioma	-1.68	0.64	no	
ko52	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.68	0.29	yes	Missense Mutation
kopn-8	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.19	1.94	yes	Missense Mutation
kp-2	pancreas	carcinoma	0.62	0.64	yes	Missense Mutation
kp-3	pancreas	carcinoma	0.79	-1.52	yes	Frame Shift Ins
kp4	pancreas	carcinoma	0.55	-3.16	no	
kpl-1	breast	carcinoma	1.02	0.96	no	
kp-n-rt-bm-1	autonomic_ganglia	neuroblastoma	0.22	0.54	no	
kp-n-si9s	autonomic_ganglia	neuroblastoma	0.51	0.55	no	
kp-n-yn	autonomic_ganglia	neuroblastoma	0.04	0.38	no	
ks-1	central_nervous_system	glioma	-1.11	0.26	no	
ku-19-19	urinary_tract	carcinoma	0.31	-0.05	no	
ku812	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.02	1.17	yes	Missense Mutation
kym-1	soft_tissue	rhabdomyosarcoma	0.35	0.24	no	
kyo-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-1.78	0.67	yes	Missense Mutation

kyse-140	oesophagus	carcinoma	0.07	1.15	yes	Missense Mutation
kyse-150	oesophagus	carcinoma	-0.97	1.10	yes	Missense Mutation
kyse-180	oesophagus	carcinoma	-1.04	0.62	no	
kyse-270	oesophagus	carcinoma	0.75	-1.25	yes	Splice Site SNP
kyse-30	oesophagus	carcinoma	-1.11	0.08	yes	Missense Mutation
kyse-410	oesophagus	carcinoma	0.22	-0.03	yes	Missense Mutation
kyse-450	oesophagus	carcinoma	0.83	1.26	yes	Missense Mutation
kyse-510	oesophagus	carcinoma	-2.09	-0.48	no	
kyse-520	oesophagus	carcinoma	0.87	0.33	yes	Splice Site SNP
kyse-70	oesophagus	carcinoma	-2.35	0.06	yes	Frame Shift Ins
l-1236	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.02	-3.16	no	
l3.3	pancreas	carcinoma	-0.81	-1.44	no	
l-363	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.58	-0.49	yes	Missense Mutation
l-428	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.56	0.62	no	
l-540	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.25	0.85	no	
lama-84	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.28	-0.92	no	
lc-1/sq-sf	lung	carcinoma	0.28	0.14	yes	Missense Mutation

lclc-103h	lung	carcinoma	-1.42	0.57	yes	Missense Mutation
lclc-97tm1	lung	carcinoma	0.46	0.53	yes	Frame Shift Del
li-7	liver	carcinoma	-0.91	-1.94	no	
lk-2	lung	carcinoma	-2.35	0.69	yes	Missense Mutation
lmsu	stomach	carcinoma	-2.35	0.81	yes	Missense Mutation
ln-18	central_nervous_system	glioma	0.62	0.20	yes	Missense Mutation
ln-229	central_nervous_system	glioma	-2.30	0.05	yes	Missense Mutation
lncap clone fgc	prostate	carcinoma	0.75	0.64	no	
loucy	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.59	0.89	yes	Missense Mutation
lou-nh91	lung	carcinoma	0.66	0.20	yes	Missense Mutation
lovo	large_intestine	carcinoma	0.80	0.36	no	
lox imvi	skin	malignant_melanoma	-2.03	0.30	no	
lp-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.61	-0.05	yes	Missense Mutation
ls 180	large_intestine	carcinoma	0.47	0.93	no	
ls1034	large_intestine	carcinoma	-0.26	0.67	yes	Missense Mutation
ls123	large_intestine	carcinoma	-0.05	1.03	yes	Missense Mutation
ls411n	large_intestine	carcinoma	0.50	-1.00	yes	Nonsense Mutation
ls513	large_intestine	carcinoma	-0.63	0.86	no	
lu65	lung	carcinoma	-2.03	-1.15	yes	Missense Mutation

lu99	lung	carcinoma	1.13	-0.50	no	
ludlu-1	lung	carcinoma	0.07	0.22	yes	Missense Mutation
lxf-289	lung	carcinoma	-0.36	0.95	yes	Missense Mutation
m059k	central_nervous_system	glioma	-1.92	0.24	yes	Missense Mutation
m-07e	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.91	1.18	no	
malme-3m	skin	malignant_melanoma	0.06	0.25	no	
mc116	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.70	1.12	yes	Missense Mutation
mcas	ovary	carcinoma	-1.03	0.97	no	
mcf7	breast	carcinoma	1.05	0.77	no	
mda pca 2b	prostate	carcinoma	0.92	0.03	no	
mda-mb-134-vi	breast	carcinoma	0.50	-0.70	no	
mda-mb-157	breast	carcinoma	0.00	-1.40	no	
mda-mb-175-vii	breast	carcinoma	0.84	-0.53	no	
mda-mb-231	breast	carcinoma	-2.00	0.28	yes	Missense Mutation
mda-mb-361	breast	carcinoma	-0.46	-0.91	yes	Nonsense Mutation
mda-mb-415	breast	carcinoma	1.13	0.56	yes	Missense Mutation
mda-mb-436	breast	carcinoma	1.20	-0.48	no	
mda-mb-453	breast	carcinoma	0.63	-2.71	no	
mda-mb-468	breast	carcinoma	0.36	1.22	no	
mdst8	large_intestine	carcinoma	0.06	0.52	no	
me-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-2.35	-0.66	no	

mec-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.38	-1.02	yes	Frame Shift Ins
mec-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.18	-0.57	no	
meg-01	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-1.21	0.69	yes	In Frame Del
mel-ho	skin	malignant_melanoma	-0.21	-0.45	no	
mel-juso	skin	malignant_melanoma	-1.80	0.47	no	
mewo	skin	malignant_melanoma	0.40	-0.38	yes	Nonsense Mutation
mfe-280	endometrium	carcinoma	0.55	-0.80	yes	Splice Site SNP
mfe-296	endometrium	carcinoma	1.07	-0.12	yes	Nonsense Mutation
mfe-319	endometrium	carcinoma	0.32	0.40	yes	Missense Mutation
mg-63	bone	osteosarcoma	0.26	-3.65	no	
mhh-call-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.39	1.30	no	
mhh-call-3	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.89	0.97	no	
mhh-call-4	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.25	1.05	no	
mhh-es-1	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	0.58	0.85	yes	In Frame Del
mhh-nb-11	autonomic_ganglia	neuroblastoma	1.04	-0.36	no	
mia paca-2	pancreas	carcinoma	0.63	1.07	yes	Missense Mutation
mino	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.35	1.14	yes	Missense Mutation

mj	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.14	1.24	no	
mkn1	stomach	carcinoma	0.29	0.63	yes	Missense Mutation
mkn-45	stomach	carcinoma	0.83	0.55	no	
mkn7	stomach	carcinoma	-0.01	1.09	yes	Missense Mutation
mkn74	stomach	carcinoma	0.44	0.97	yes	Missense Mutation
ml-1	thyroid	carcinoma	0.47	-2.20	no	
mm1-s	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.03	0.47	no	
molm-13	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.20	1.29	no	
molm-16	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.45	1.49	yes	Missense Mutation
molm-6	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.82	1.02	yes	Missense Mutation
molp-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.32	0.49	yes	Missense Mutation
molp-8	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.14	0.17	no	
molt-13	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.46	0.58	no	
molt-16	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.03	1.13	yes	Missense Mutation
molt-4	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.86	0.05	no	
mono-mac-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.11	0.33	no	
mono-mac-6	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.17	0.61	no	

mor/cpr	lung	carcinoma	0.58	-1.40	yes	Frame Shift Del
motn-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.21	0.11	yes	Splice Site SNP
mpp 89	pleura	mesothelioma	0.62	-3.65	no	
msto-211h	pleura	mesothelioma	0.38	-0.27	no	
mutz-5	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.11	1.03	no	
mv-4-11	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.04	1.25	no	
nalm-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.80	1.18	no	
nalm-19	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.65	1.38	no	
nalm-6	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.65	1.00	no	
nb-1	autonomic_ganglia	neuroblastoma	0.19	0.79	no	
nb-4	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.09	0.79	yes	Missense Mutation
nci-h1048	lung	carcinoma	0.20	-0.09	yes	Missense Mutation
nci-h1092	lung	carcinoma	-2.35	-1.70	yes	Splice Site SNP
nci-h1105	lung	carcinoma	-2.35	0.89	yes	Missense Mutation
nci-h1155	lung	carcinoma	0.01	-0.20	no	
nci-h1184	lung	carcinoma	-2.13	0.42	yes	Missense Mutation
nci-h1299	lung	carcinoma	0.17	0.58	no	
nci-h1339	lung	carcinoma	-2.13	-1.13	yes	Nonsense Mutation
nci-h1341	lung	carcinoma	0.49	0.13	no	

nci-h1355	lung	carcinoma	0.41	-0.05	yes	Missense Mutation
nci-h1373	lung	carcinoma	-2.03	-1.88	yes	Nonsense Mutation
nci-h1385	lung	carcinoma	-1.41	-0.77	no	
nci-h1395	lung	carcinoma	-0.03	-0.77	no	
nci-h1435	lung	carcinoma	-0.77	0.08	yes	Missense Mutation
nci-h1436	lung	carcinoma	0.94	0.61	yes	Missense Mutation
nci-h1437	lung	carcinoma	0.74	-0.55	yes	Missense Mutation
nci-h146	lung	carcinoma	-0.32	-0.89	no	
nci-h1563	lung	carcinoma	0.92	0.18	no	
nci-h1568	lung	carcinoma	0.13	0.09	yes	Missense Mutation
nci-h1573	lung	carcinoma	0.80	-0.15	yes	Missense Mutation
nci-h1581	lung	carcinoma	0.54	-3.24	yes	Nonsense Mutation
nci-h1618	lung	carcinoma	-2.35	0.10	yes	Missense Mutation
nci-h1623	lung	carcinoma	0.01	-0.21	yes	Missense Mutation
nci-h1648	lung	carcinoma	0.21	-0.94	yes	Frame Shift Ins
nci-h1650	lung	carcinoma	0.64	-1.20	yes	Splice Site SNP
nci-h1651	lung	carcinoma	-0.15	0.36	yes	Missense Mutation
nci-h1666	lung	carcinoma	-0.71	0.07	no	

nci-h1693	lung	carcinoma	0.83	-0.85	yes	Splice Site SNP
nci-h1694	lung	carcinoma	-0.11	0.00	yes	Splice Site SNP
nci-h1703	lung	carcinoma	0.33	-0.73	yes	Splice Site SNP
nci-h1734	lung	carcinoma	-0.61	0.18	yes	Missense Mutation
nci-h1755	lung	carcinoma	-1.78	-0.13	no	
nci-h1781	lung	carcinoma	0.77	1.01	yes	Missense Mutation
nci-h1792	lung	carcinoma	-0.27	-2.19	yes	Splice Site SNP
nci-h1793	lung	carcinoma	0.37	-0.46	yes	Nonsense Mutation
nci-h1836	lung	carcinoma	-2.35	0.47	yes	Missense Mutation
nci-h1838	lung	carcinoma	-0.10	0.63	yes	Missense Mutation
nci-h1869	lung	carcinoma	0.53	0.04	yes	Missense Mutation
nci-h1876	lung	carcinoma	0.30	0.34	yes	Missense Mutation
nci-h1915	lung	carcinoma	-2.03	-1.78	yes	Nonsense Mutation
nci-h1930	lung	carcinoma	0.49	0.09	yes	Missense Mutation
nci-h1944	lung	carcinoma	-0.11	-0.27	no	
nci-h196	lung	carcinoma	0.39	0.29	yes	Missense Mutation
nci-h1963	lung	carcinoma	-0.24	0.47	yes	Missense Mutation

nci-h1975	lung	carcinoma	0.49	1.13	no	
nci-h2009	lung	carcinoma	-0.16	0.89	yes	Missense Mutation
nci-h2023	lung	carcinoma	0.40	-0.13	yes	Missense Mutation
nci-h2029	lung	carcinoma	-0.34	-0.31	yes	Missense Mutation
nci-h2030	lung	carcinoma	-2.17	0.43	yes	Missense Mutation
nci-h2052	pleura	mesothelioma	-0.38	0.12	no	
nci-h2066	lung	carcinoma	0.49	0.58	yes	Missense Mutation
nci-h2081	lung	carcinoma	-0.19	-3.65	no	
nci-h2085	lung	carcinoma	0.51	0.75	no	
nci-h2087	lung	carcinoma	0.95	0.84	yes	Missense Mutation
nci-h209	lung	carcinoma	-0.54	-1.30	yes	Splice Site SNP
nci-h2106	lung	carcinoma	0.31	0.72	yes	Missense Mutation
nci-h211	lung	carcinoma	-0.28	0.12	yes	Missense Mutation
nci-h2110	lung	carcinoma	0.23	0.69	yes	Missense Mutation
nci-h2122	lung	carcinoma	-0.29	0.28	yes	Missense Mutation
nci-h2126	lung	carcinoma	0.26	-1.30	no	
nci-h2141	lung	carcinoma	-0.80	-1.49	yes	Nonsense Mutation
nci-h2170	lung	carcinoma	-2.00	0.75	yes	Missense Mutation

nci-h2171	lung	carcinoma	0.22	-0.88	yes	Nonsense Mutation
nci-h2172	lung	carcinoma	0.11	-1.56	no	
nci-h2196	lung	carcinoma	-2.21	-0.07	yes	Missense Mutation
nci-h2227	lung	carcinoma	-2.35	0.38	yes	Splice Site SNP
nci-h2228	lung	carcinoma	0.82	-1.33	yes	Nonsense Mutation
nci-h226	lung	carcinoma	0.20	0.62	no	
nci-h2286	lung	carcinoma	1.01	-1.56	yes	Nonsense Mutation
nci-h2291	lung	carcinoma	1.03	-0.24	yes	Missense Mutation
nci-h23	lung	carcinoma	0.38	0.33	yes	Missense Mutation
nci-h2342	lung	carcinoma	-0.14	0.79	yes	Missense Mutation
nci-h2347	lung	carcinoma	0.44	-1.70	no	
nci-h2405	lung	carcinoma	0.78	0.38	no	
nci-h2444	lung	carcinoma	0.68	0.86	yes	Missense Mutation
nci-h2452	pleura	mesothelioma	0.73	-3.65	no	
nci-h28	pleura	mesothelioma	0.92	0.46	no	
nci-h322	lung	carcinoma	-0.25	0.30	no	
nci-h358	lung	carcinoma	0.49	-2.59	no	
nci-h441	lung	carcinoma	1.14	0.57	yes	Missense Mutation
nci-h446	lung	carcinoma	-2.35	0.67	yes	Missense Mutation
nci-h460	lung	carcinoma	0.15	0.07	no	
nci-h508	large_intestine	carcinoma	0.80	0.61	no	

nci-h510	lung	carcinoma	-1.22	0.16	yes	Missense Mutation
nci-h520	lung	carcinoma	-0.03	-1.82	yes	Nonsense Mutation
nci-h522	lung	carcinoma	0.42	-1.58	yes	Frame Shift Del
nci-h524	lung	carcinoma	-0.27	-0.13	yes	Missense Mutation
nci-h526	lung	carcinoma	0.27	0.23	yes	Splice Site SNP
nci-h596	lung	carcinoma	0.97	0.63	yes	Missense Mutation
nci-h647	lung	carcinoma	-0.20	-0.26	yes	Splice Site SNP
nci-h650	lung	carcinoma	0.58	-0.59	yes	Missense Mutation
nci-h660	prostate	carcinoma	0.22	-3.65	no	
nci-h661	lung	carcinoma	0.13	0.14	yes	Missense Mutation
nci-h69	lung	carcinoma	-1.40	-1.75	yes	Nonsense Mutation
nci-h716	large_intestine	carcinoma	0.82	-1.64	yes	Missense Mutation
nci-h727	lung	carcinoid-endocrine_tumour	-1.43	0.64	yes	In Frame Ins
nci-h747	large_intestine	carcinoma	0.97	0.62	yes	Missense Mutation
nci-h810	lung	carcinoma	-2.35	-1.51	yes	Nonsense Mutation
nci-h82	lung	carcinoma	0.26	-1.29	no	
nci-h838	lung	carcinoma	0.43	-1.44	no	
nci-h841	lung	carcinoma	0.65	1.58	yes	Missense Mutation

nci-h854	lung	carcinoma	0.00	0.62	yes	Missense Mutation
nci-h889	lung	carcinoma	0.09	0.02	yes	Missense Mutation
nci-h929	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.73	0.15	no	
nci-n87	stomach	carcinoma	0.45	0.46	yes	Missense Mutation
nco2	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.85	0.86	yes	Missense Mutation
nh-6	autonomic_ganglia	neuroblastoma	0.24	0.02	no	
nih:ovcar-3	ovary	carcinoma	0.98	0.51	yes	Missense Mutation
nmc-g1	central_nervous_system	glioma	-2.17	0.01	no	
nomo-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-2.35	-1.12	no	
nu-dhl-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.87	1.29	yes	Missense Mutation
nu-dul-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.19	1.35	yes	Missense Mutation
nugc-2	stomach	carcinoma	-0.91	1.43	no	
nugc-3	stomach	carcinoma	0.66	1.31	yes	Missense Mutation
nugc-4	stomach	carcinoma	0.43	0.95	no	
oaw28	ovary	carcinoma	0.66	-1.91	yes	Frame Shift Del
oaw42	ovary	carcinoma	1.28	0.78	no	
oc 314	ovary	carcinoma	0.99	0.07	no	
oc 316	ovary	carcinoma	0.76	0.11	no	
oci-aml2	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-1.60	1.03	no	

oci-aml3	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.73	1.41	no	
oci-aml5	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.61	0.95	no	
oci-ly-19	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.35	1.96	no	
oci-ly3	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.21	0.69	no	
oci-m1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-1.31	0.57	yes	Splice Site SNP
ocum-1	stomach	carcinoma	0.75	0.78	no	
oe19	oesophagus	carcinoma	0.58	-0.81	yes	Frame Shift Ins
oe33	oesophagus	other	-2.13	-0.37	yes	Missense Mutation
ons-76	central_nervous_system	primitive_neuroectodermal_tumour-medulloblastoma	0.83	0.18	no	
opm-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.15	0.91	yes	Missense Mutation
oums-23	large_intestine	carcinoma	0.88	0.47	yes	Missense Mutation
oums-27	bone	chondrosarcoma	0.75	-1.35	no	
ov56	ovary	carcinoma	0.71	-1.52	no	
ov7	ovary	carcinoma	0.49	0.41	no	
ov-90	ovary	carcinoma	-0.30	0.22	yes	Missense Mutation
ovcar-4	ovary	carcinoma	0.80	0.43	yes	Missense Mutation
ovcar-8	ovary	carcinoma	0.80	0.22	yes	Splice Site SNP
ovk18	ovary	carcinoma	0.93	0.14	yes	Frame Shift Del

ovkate	ovary	carcinoma	0.19	-0.19	yes	Missense Mutation
ovmana	ovary	carcinoma	0.52	-0.93	no	
ovsaho	ovary	carcinoma	1.27	-0.62	yes	Nonsense Mutation
ovtoko	ovary	carcinoma	0.91	0.49	no	
p12-ichikawa	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.19	1.39	yes	Missense Mutation
p31/fuj	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-2.13	0.09	yes	Nonsense Mutation
p3hr-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.16	0.03	yes	Nonsense Mutation
panc 02.03	pancreas	carcinoma	-0.05	-0.09	yes	Missense Mutation
panc 02.13	pancreas	carcinoma	-0.35	-0.07	yes	Missense Mutation
panc 03.27	pancreas	carcinoma	-0.19	-1.01	no	
panc 04.03	pancreas	carcinoma	0.66	0.29	yes	Missense Mutation
panc 05.04	pancreas	carcinoma	1.01	-0.03	no	
panc 08.13	pancreas	carcinoma	-0.40	-2.17	no	
panc 10.05	pancreas	carcinoma	0.86	-0.19	yes	Missense Mutation
panc-1	pancreas	carcinoma	0.70	0.01	no	
pa-tu-8902	pancreas	carcinoma	-0.01	1.30	yes	Missense Mutation
pa-tu-8988s	pancreas	carcinoma	0.79	0.44	no	
pa-tu-8988t	pancreas	carcinoma	0.84	1.30	no	
pc-14	lung	carcinoma	0.66	1.39	yes	Missense Mutation
pc-3	prostate	carcinoma	0.24	-1.54	yes	Frame Shift Del

pcm6	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.94	0.36	yes	Missense Mutation
pe/ca-pj15	upper_aerodigestive_tract	carcinoma	-1.34	0.62	yes	Missense Mutation
pe/ca-pj34 (clone c12)	upper_aerodigestive_tract	carcinoma	-2.09	1.61	yes	Missense Mutation
pe/ca-pj41 (clone d2)	upper_aerodigestive_tract	carcinoma	0.17	-0.94	yes	Splice Site SNP
pe/ca-pj49	upper_aerodigestive_tract	carcinoma	-0.53	-3.65	no	
peer	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.62	0.17	yes	Missense Mutation
pf-382	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.55	0.27	yes	Missense Mutation
pfeiffer	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.56	-3.65	no	
pk-1	pancreas	carcinoma	0.45	0.35	yes	Missense Mutation
pk-45h	pancreas	carcinoma	0.51	-0.36	no	
pk-59	pancreas	carcinoma	0.11	-1.05	no	
pl-21	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.23	0.09	yes	Frame Shift Del
pl45	pancreas	carcinoma	0.64	-0.04	no	
plc/prf/5	liver	carcinoma	0.60	-0.38	no	
psn1	pancreas	carcinoma	0.86	-0.38	yes	Missense Mutation
qgp-1	pancreas	carcinoma	-0.12	-1.22	no	
raji	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.40	0.60	yes	Missense Mutation
rcc10rgb	kidney	carcinoma	1.04	0.38	no	
rch-acv	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.84	1.21	no	

rcm-1	large_intestine	carcinoma	-2.35	-1.44	yes	Nonsense Mutation
rd	soft_tissue	rhabdomyosarcoma	0.29	0.44	yes	Missense Mutation
rd-es	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	0.71	0.84	no	
rec-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-0.14	0.57	yes	Nonsense Mutation
reh	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.73	1.17	no	
rerf-gc-1b	stomach	carcinoma	1.04	-1.22	yes	Nonsense Mutation
rerf-lc-ad1	lung	carcinoma	0.66	0.41	yes	Missense Mutation
rerf-lc-ad2	lung	carcinoma	0.55	0.45	yes	Missense Mutation
rerf-lc-ai	lung	carcinoma	0.57	-1.34	no	
rerf-lc-kj	lung	carcinoma	0.69	-1.53	yes	Missense Mutation
rerf-lc-ms	lung	carcinoma	0.45	-1.75	yes	Frame Shift Del
rerf-lc-sq1	lung	carcinoma	-1.19	-1.08	no	
rh-41	soft_tissue	rhabdomyosarcoma	0.05	-2.29	yes	Frame Shift Del
ri-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.38	0.66	yes	Nonsense Mutation
rkn	soft_tissue	leiomyosarcoma	0.93	0.10	yes	Missense Mutation
rko	large_intestine	carcinoma	0.60	-0.91	no	
rl	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.27	0.32	yes	Missense Mutation

rl95-2	endometrium	carcinoma	0.93	0.26	yes	In Frame Del
rmg-i	ovary	carcinoma	0.37	-0.01	no	
rmug-s	ovary	carcinoma	0.68	0.93	yes	Missense Mutation
rpmi 8226	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.01	-0.04	yes	Missense Mutation
rpmi-7951	skin	malignant_melanoma	-0.26	-2.20	yes	Nonsense Mutation
rpmi-8402	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.53	1.13	yes	Missense Mutation
rs4-11	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.18	0.81	no	
rt-112	urinary_tract	carcinoma	0.75	0.75	yes	Nonsense Mutation
rt112/84	urinary_tract	carcinoma	1.08	0.53	yes	Missense Mutation
rt4	urinary_tract	carcinoma	0.59	0.22	no	
rvh-421	skin	malignant_melanoma	0.65	0.00	no	
s-117	soft_tissue	sarcoma	-2.35	-1.58	yes	Nonsense Mutation
sbc-5	lung	carcinoma	1.13	0.41	yes	Missense Mutation
scaber	urinary_tract	carcinoma	-0.43	0.14	yes	Missense Mutation
scc-15	upper_aerodigestive_tract	carcinoma	-0.09	-1.14	yes	Splice Site SNP
scc-25	upper_aerodigestive_tract	carcinoma	0.60	-2.07	yes	Frame Shift Del
scc-4	upper_aerodigestive_tract	carcinoma	0.44	0.75	yes	Missense Mutation
scc-9	upper_aerodigestive_tract	carcinoma	-0.72	-1.27	no	

sclc-21h	lung	carcinoma	-0.25	0.16	yes	Missense Mutation
sem	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.13	0.65	yes	Missense Mutation
set-2	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-0.21	0.58	yes	Missense Mutation
sf126	central_nervous_system	glioma	-2.35	-1.88	no	
sf-295	central_nervous_system	glioma	-0.54	0.11	yes	Missense Mutation
sh-10-tc	stomach	carcinoma	-0.04	-0.01	yes	Missense Mutation
sh-4	skin	malignant_melanoma	-0.89	-0.51	no	
shp-77	lung	carcinoma	-2.35	1.17	no	
sh-sy5y	autonomic_ganglia	neuroblastoma	0.34	0.02	no	
sig-m5	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-2.35	1.05	no	
sima	autonomic_ganglia	neuroblastoma	0.69	0.57	no	
sjrh30	soft_tissue	rhabdomyosarcoma	-0.41	-0.02	yes	Missense Mutation
sjsa-1	bone	osteosarcoma	-0.11	0.28	no	
sk-br-3	breast	carcinoma	-0.04	0.27	yes	Missense Mutation
sk-co-1	large_intestine	carcinoma	-2.35	0.67	no	
sk-es-1	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	0.72	1.10	yes	Missense Mutation
sk-hep-1	liver	carcinoma	0.44	0.67	no	
sk-lms-1	soft_tissue	sarcoma	-1.61	0.59	yes	Missense Mutation
sk-lu-1	lung	carcinoma	-1.02	0.45	yes	Missense Mutation

skm-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	1.03	0.61	yes	Missense Mutation
sk-mel-1	skin	malignant_melanoma	0.63	-0.67	no	
sk-mel-2	skin	malignant_melanoma	0.13	-0.57	yes	Missense Mutation
sk-mel-24	skin	malignant_melanoma	-2.35	0.26	no	
sk-mel-28	skin	malignant_melanoma	-0.03	-0.18	yes	Missense Mutation
sk-mel-3	skin	malignant_melanoma	-0.53	0.27	yes	Missense Mutation
sk-mel-30	skin	malignant_melanoma	-1.82	-0.76	yes	Frame Shift Del
sk-mel-31	skin	malignant_melanoma	-0.77	-0.04	no	
sk-mel-5	skin	malignant_melanoma	0.19	-0.23	no	
sk-mes-1	lung	carcinoma	0.94	-1.27	yes	Nonsense Mutation
sk-mm-2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.03	0.53	no	
sk-n-as	autonomic_ganglia	neuroblastoma	0.89	-3.65	no	
sk-n-be(2)	autonomic_ganglia	neuroblastoma	0.50	0.41	no	
sk-n-dz	autonomic_ganglia	neuroblastoma	-0.39	1.14	no	
sk-n-fi	autonomic_ganglia	neuroblastoma	0.58	0.08	yes	Missense Mutation
sk-n-mc	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	0.34	0.29	no	
sk-n-sh	autonomic_ganglia	neuroblastoma	-0.15	0.31	no	
sk-ut-1	soft_tissue	leiomyosarcoma	0.43	0.60	yes	Missense Mutation
sng-m	endometrium	carcinoma	0.93	0.94	no	
snu-1	stomach	carcinoma	0.91	0.04	no	

snu-1040	large_intestine	carcinoma	1.02	0.18	yes	Missense Mutation
snu-1076	upper_aerodigestive_tract	carcinoma	0.64	-1.11	no	
snu-1077	endometrium	carcinoma	0.66	0.63	yes	Missense Mutation
snu-1079	biliary_tract	carcinoma	1.11	0.98	no	
snu-1105	central_nervous_system	glioma	-1.41	0.01	yes	Missense Mutation
snu-119	ovary	carcinoma	0.78	-0.05	yes	Missense Mutation
snu-1196	biliary_tract	carcinoma	0.60	1.54	yes	Missense Mutation
snu-1214	upper_aerodigestive_tract	carcinoma	0.44	0.41	yes	Missense Mutation
snu-1272	kidney	carcinoma	0.98	0.39	no	
snu-16	stomach	carcinoma	0.62	1.02	no	
snu-175	large_intestine	carcinoma	0.24	0.91	no	
snu-182	liver	carcinoma	0.88	0.42	yes	Missense Mutation
snu-201	central_nervous_system	glioma	-0.83	0.93	yes	Missense Mutation
snu-213	pancreas	carcinoma	0.51	0.60	yes	Missense Mutation
snu-216	stomach	carcinoma	0.60	0.95	yes	Missense Mutation
snu-245	biliary_tract	carcinoma	0.98	1.03	yes	Missense Mutation
snu-283	large_intestine	carcinoma	1.00	-3.65	no	
snu-308	biliary_tract	carcinoma	0.47	0.08	yes	Missense Mutation
snu-324	pancreas	carcinoma	0.68	0.07	no	

snu-387	liver	carcinoma	0.23	-1.23	yes	Nonsense Mutation
snu-398	liver	carcinoma	1.04	-1.66	no	
snu-407	large_intestine	carcinoma	1.02	0.75	no	
snu-410	pancreas	carcinoma	0.99	0.30	yes	Missense Mutation
snu-423	liver	carcinoma	-0.29	0.35	yes	Splice Site SNP
snu-449	liver	carcinoma	1.17	0.17	yes	Missense Mutation
snu-46	upper_aerodigestive_tract	carcinoma	1.03	0.81	yes	Missense Mutation
snu-466	central_nervous_system	glioma	0.73	0.53	no	
snu-475	liver	carcinoma	0.01	0.57	yes	Missense Mutation
snu-478	biliary_tract	carcinoma	0.63	-1.82	yes	Frame Shift Ins
snu-5	stomach	carcinoma	0.43	-0.16	yes	Splice Site SNP
snu-503	large_intestine	carcinoma	1.01	0.56	yes	Missense Mutation
snu-520	stomach	carcinoma	0.60	0.25	no	
snu-61	large_intestine	carcinoma	1.02	0.97	no	
snu-620	stomach	carcinoma	-1.76	0.51	no	
snu-626	central_nervous_system	glioma	0.12	0.47	yes	Missense Mutation
snu-738	central_nervous_system	glioma	-2.35	-1.47	yes	Nonsense Mutation
snu-761	liver	carcinoma	1.12	-1.35	no	
snu-840	ovary	carcinoma	0.05	-0.50	no	

snu-878	liver	carcinoma	0.04	-0.02	yes	Missense Mutation
snu-899	upper_aerodigestive_tract	carcinoma	0.80	-0.35	no	
snu-c1	large_intestine	carcinoma	0.82	-2.14	no	
snu-c2a	large_intestine	carcinoma	0.90	-0.02	yes	Missense Mutation
snu-c4	large_intestine	carcinoma	0.75	0.72	yes	Missense Mutation
snu-c5	large_intestine	carcinoma	-2.35	0.72	yes	Missense Mutation
sq-1	lung	carcinoma	-2.35	-0.98	no	
sr-786	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.30	0.10	yes	Missense Mutation
st486	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.11	1.18	yes	Missense Mutation
su.86.86	pancreas	carcinoma	-0.74	0.18	yes	Missense Mutation
su-dhl-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.00	0.85	no	
su-dhl-10	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.66	0.23	yes	Splice Site SNP
su-dhl-4	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.31	1.23	yes	Missense Mutation
su-dhl-5	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.16	1.20	no	
su-dhl-6	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.25	1.05	yes	Missense Mutation
su-dhl-8	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.35	1.77	yes	Missense Mutation
suit-2	pancreas	carcinoma	0.64	0.35	no	

sup-b15	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.91	0.84	no	
sup-hd1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.35	0.92	no	
sup-m2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-2.35	0.47	no	
sup-t1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.48	0.95	no	
sup-t11	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.65	-1.03	yes	Nonsense Mutation
sw 1088	central_nervous_system	glioma	-2.35	0.50	yes	Missense Mutation
sw 1271	lung	carcinoma	0.89	0.92	yes	Missense Mutation
sw 1353	bone	chondrosarcoma	-0.37	-0.10	yes	Missense Mutation
sw 1573	lung	carcinoma	0.44	0.57	no	
sw 1783	central_nervous_system	glioma	-0.80	0.39	yes	Missense Mutation
sw 1990	pancreas	carcinoma	0.36	0.46	no	
sw 780	urinary_tract	carcinoma	0.39	0.34	no	
sw 900	lung	carcinoma	0.83	-1.00	yes	Nonsense Mutation
sw1116	large_intestine	carcinoma	0.64	0.71	yes	Missense Mutation
sw1417	large_intestine	carcinoma	0.55	-1.53	yes	Frame Shift Del
sw1463	large_intestine	carcinoma	0.23	0.39	yes	Missense Mutation
sw-1710	urinary_tract	carcinoma	0.43	0.51	yes	Missense Mutation

sw403	large_intestine	carcinoma	0.81	-0.93	yes	Nonsense Mutation
sw48	large_intestine	carcinoma	-1.92	1.08	no	
sw480	large_intestine	carcinoma	-0.07	0.77	no	
sw579	thyroid	carcinoma	0.30	0.99	yes	Missense Mutation
sw620	large_intestine	carcinoma	-2.30	0.71	no	
sw837	large_intestine	carcinoma	0.68	0.75	yes	Missense Mutation
sw948	large_intestine	carcinoma	0.54	-1.07	no	
t.t	oesophagus	carcinoma	0.43	-0.01	yes	Nonsense Mutation
t1-73	bone	osteosarcoma	0.31	-0.11	no	
t24	urinary_tract	carcinoma	0.67	-1.58	no	
t3m-4	pancreas	carcinoma	0.16	0.17	yes	Missense Mutation
t-47d	breast	carcinoma	0.90	0.28	yes	Missense Mutation
t84	large_intestine	carcinoma	-1.52	-0.95	yes	Splice Site SNP
t98g	central_nervous_system	glioma	0.13	0.94	yes	Missense Mutation
tall-1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.32	1.03	no	
tc-71	bone	ewings_sarcoma-peripheral_primitive_neuroectodermal_tumour	0.50	-1.87	yes	Nonsense Mutation
tcc-pan2	pancreas	carcinoma	1.04	-1.37	yes	Splice Site SNP
te 441.t	soft_tissue	rhabdomyosarcoma	0.63	0.83	no	
te 617.t	soft_tissue	rhabdomyosarcoma	0.88	-0.32	no	

te-1	oesophagus	carcinoma	0.61	0.96	yes	Missense Mutation
te-10	oesophagus	carcinoma	0.62	0.40	yes	Missense Mutation
te-11	oesophagus	carcinoma	-0.61	0.43	no	
te-14	oesophagus	carcinoma	0.09	-1.55	yes	Nonsense Mutation
te-15	oesophagus	carcinoma	-1.94	-0.58	no	
te-4	oesophagus	carcinoma	-1.76	0.31	yes	Missense Mutation
te-5	oesophagus	carcinoma	0.29	0.56	yes	Missense Mutation
te-6	oesophagus	carcinoma	0.68	0.91	yes	Missense Mutation
te-8	oesophagus	carcinoma	0.61	0.37	yes	Missense Mutation
ten	endometrium	carcinoma	0.84	-0.72	yes	Nonsense Mutation
tf-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-2.35	-0.64	yes	Frame Shift Del
tgbc11tkb	stomach	carcinoma	-2.35	0.53	yes	Missense Mutation
thp-1	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	0.17	-1.71	yes	Frame Shift Del
tm-31	central_nervous_system	glioma	-1.52	0.12	yes	Missense Mutation
to 175.t	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.42	-0.12	no	
toledo	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	0.25	-1.91	yes	Frame Shift Del
tov-112d	ovary	carcinoma	0.87	1.41	no	
tov-21g	ovary	carcinoma	1.39	0.48	no	

tt	thyroid	carcinoma	-2.35	-0.47	no	
tt2609-c02	thyroid	carcinoma	0.36	0.77	yes	Missense Mutation
tuhr10tkb	kidney	carcinoma	0.43	0.32	no	
tuhr14tkb	kidney	carcinoma	0.81	-0.03	no	
tuhr4tkb	kidney	carcinoma	0.76	0.35	no	
tyk-nu	ovary	carcinoma	-1.20	-0.17	yes	Missense Mutation
u-138 mg	central_nervous_system	glioma	0.25	0.04	yes	Missense Mutation
u-2 os	bone	osteosarcoma	0.17	-0.10	no	
u-251 mg	central_nervous_system	glioma	-1.69	0.78	no	
u266b1	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.25	-0.40	yes	Missense Mutation
u-87 mg	central_nervous_system	glioma	-2.30	-0.28	no	
u-937	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	-1.07	-1.90	yes	Splice Site SNP
uacc-257	skin	malignant_melanoma	0.23	-0.08	no	
uacc-62	skin	malignant_melanoma	-1.38	-0.25	no	
uacc-812	breast	carcinoma	0.48	0.14	no	
uacc-893	breast	carcinoma	-2.30	-1.07	yes	Nonsense Mutation
um-uc-3	urinary_tract	carcinoma	0.00	0.23	no	
ut-7	haematopoietic_and_lymphoid_tissue	haematopoietic_neoplasm	-1.38	-0.39	yes	Splice Site SNP
vcap	prostate	carcinoma	-0.07	0.25	yes	Missense Mutation
vm-cub1	urinary_tract	carcinoma	-0.51	0.21	yes	Missense Mutation
vmrc-rcw	kidney	carcinoma	0.22	-0.81	no	

vmrc-rcz	kidney	carcinoma	0.35	-1.41	no	
wm-115	skin	malignant_melanoma	-1.78	0.29	no	
wm1799	skin	malignant_melanoma	-2.35	0.41	no	
wm-266-4	skin	malignant_melanoma	-1.41	0.54	no	
wm-793	skin	malignant_melanoma	-0.70	0.34	no	
wm-88	skin	malignant_melanoma	-0.28	0.39	no	
wm-983b	skin	malignant_melanoma	-2.35	0.75	yes	Missense Mutation
wsu-dlcl2	haematopoietic_and_lymphoid_tissue	lymphoid_neoplasm	1.55	1.42	yes	Missense Mutation
yapc	pancreas	carcinoma	1.37	0.77	yes	Missense Mutation
yd-10b	upper_aerodigestive_tract	carcinoma	0.28	-1.54	yes	Nonsense Mutation
yd-15	salivary_gland	carcinoma	-0.11	0.59	yes	Missense Mutation
yd-38	upper_aerodigestive_tract	carcinoma	-2.35	-1.24	yes	Nonsense Mutation
yd-8	upper_aerodigestive_tract	carcinoma	0.96	0.99	no	
yh-13	central_nervous_system	glioma	0.60	0.06	no	
ykg1	central_nervous_system	glioma	0.36	0.69	yes	In Frame Del
ymb-1	breast	carcinoma	0.96	-0.54	no	
zr-75-1	breast	carcinoma	0.77	-0.55	no	
zr-75-30	breast	carcinoma	0.18	0.25	no	

Table A2. *TP53* (exons 3-11) sequence in T98/EV, T98/shRNA, U87MG and A172 GBM cell lines. The point mutations are indicated in bold red. Heterozygous single nucleotide polymorphism is specified with the alternate bases separated by a slash.

U87MG
ACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCGTCCCAAGCAATGGATGATTTG ATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGAT GAAGCTCCCAGAATGCCAGAGGCTGCTCCCCGCGTGGCCCCTGCACCAGCAGCT CCTACACCGGCGGCCCCTGCACCAGCCCCCTCCTGGCCCCTGTCATCTTCTGTCC CTTCCCAGAAAACCTACCAGGGCAGCTACGGTTTCCGTCTGGGCTTCTTGCATTC TGGGACAGCCAAGTCTGTGACTTGCACGTACTCCCCTGCCCTCAACAAGATGTTT TGCCAACTGGCCAAGACCTGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCGC CCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAGCACATGACGG AGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTGG CCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGA TGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAGTTG GCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGGG CGGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGTGG TAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGA GACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGA GCTGCCCCCAGGGAGCACTAAGCGAGCACTGCCCAACAACACCAGCTCCTCTCC CCAGCCAAAGAAGAAACCACTGGATGGAGAATATTTCACCCTTCAGATCCGTGG GCGTGAGCGCTTCGAGATGTTCCGAGAGCTGAATGAGGCCTTGGAACTCAAGGA TGCCCAGGCTGGGAAGGAGCCAGGGGGGAGCAGGGCTCACTCCAGCCACCTGA AGTCCAAAAAGGGTCAGTCTACCTCCCGCCATAAAAAACTCATGTTCAAGACAG AAGGGCCTGACTCAGACTG
T98G
ACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCGTCCCAAGCAATGGATGATTTG ATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGAT GAAGCTCCCAGAATGCCAGAGGCTGCTCCCCGCGTGGCCCCTGCACCAGCAGCT CCTACACCGGCGGCCCCTGCACCAGCCCCCTCCTGGCCCCTGTCATCTTCTGTCC CTTCCCAGAAAACCTACCAGGGCAGCTACGGTTTCCGTCTGGGCTTCTTGCATTC TGGGACAGCCAAGTCTGTGACTTGCACGTACTCCCCTGCCCTCAACAAGATGTTT TGCCAACTGGCCAAGACCTGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCGC CCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAGCACATGACGG AGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTGG CCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGA TGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAGTTG GCTCTGACTGTACCACCATCCACTACAACTACAT**A**TGTAACAGTTCCTGCATGGG CGGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGTGG TAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGA GACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGA GCTGCCCCCAGGGAGCACTAAGCGAGCACTGCCCAACAACACCAGCTCCTCTCC CCAGCCAAAGAAGAAACCACTGGATGGAGAATATTTCACCCTTCAGATCCGTGG

GCGTGAGCGCTTCGAGATGTTCCGAGAGCTGAATGAGGCCTTGGAACTCAAGGA
TGCCCAGGCTGGGAAGGAGCCAGGGGGGAGCAGGGCTCACTCCAGCCACCTGA
AGTCCAAAAAGGGTCAGTCTACCTCCCGCCATAAAAAACTCATGTTCAAGACAG
AAGGGCCTGACTCAGACTG

T98/EV

ACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCGTCCCAAGCAATGGATGATTTG
ATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGAT
GAAGCTCCCAGAATGCCAGAGGCTGCTCCCCGCGTGGCCCCTGCACCAGCAGCT
CCTACACCGGCGGCCCCTGCACCAGCCCCCTCCTGGCCCCTGTCATCTTCTGTCC
CTTCCCAGAAAACCTACCAGGGCAGCTACGGTTTCCGTCTGGGCTTCTTGCATTC
TGGGACAGCCAAGTCTGTGACTTGCACGTACTCCCCTGCCCTCAACAAGATGTTT
TGCCAACTGGCCAAGACCTGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCGC
CCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAGCACATGACGG
AGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTGG
CCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGA
TGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAGTTG
GCTCTGACTGTACCACCATCCACTACAACTACAT**A**TGTAACAGTTCCTGCATGGG
CGGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGTGG
TAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGA
GACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGA
GCTGCCCCCAGGGAGCACTAAGCGAGCACTGCCCAACAACACCAGCTCCTCTCC
CCAGCCAAAGAAGAAACCACTGGATGGAGAATATTTCACCCTTCAGATCCGTGG
GCGTGAGCGCTTCGAGATGTTCCGAGAGCTGAATGAGGCCTTGGAACTCAAGGA
TGCCCAGGCTGGGAAGGAGCCAGGGGGGAGCAGGGCTCACTCCAGCCACCTGA
AGTCCAAAAAGGGTCAGTCTACCTCCCGCCATAAAAAACTCATGTTCAAGACAG
AAGGGCCTGACTCAGACTG

T98/shRNA

ACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCGTCCCAAGCAATGGATGATTTG
ATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGAT
GAAGCTCCCAGAATGCCAGAGGCTGCTCCCCGCGTGGCCCCTGCACCAGCAGCT
CCTACACCGGCGGCCCCTGCACCAGCCCCCTCCTGGCCCCTGTCATCTTCTGTCC
CTTCCCAGAAAACCTACCAGGGCAGCTACGGTTTCCGTCTGGGCTTCTTGCATTC
TGGGACAGCCAAGTCTGTGACTTGCACGTACTCCCCTGCCCTCAACAAGATGTTT
TGCCAACTGGCCAAGACCTGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCGC
CCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAGCACATGACGG
AGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTGG
CCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGGA
TGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAGTTG
GCTCTGACTGTACCACCATCCACTACAACTACAT**A**TGTAACAGTTCCTGCATGGG
CGGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGTGG
TAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAGA
GACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACGA
GCTGCCCCCAGGGAGCACTAAGCGAGCACTGCCCAACAACACCAGCTCCTCTCC
CCAGCCAAAGAAGAAACCACTGGATGGAGAATATTTCACCCTTCAGATCCGTGG
GCGTGAGCGCTTCGAGATGTTCCGAGAGCTGAATGAGGCCTTGGAACTCAAGGA
TGCCCAGGCTGGGAAGGAGCCAGGGGGGAGCAGGGCTCACTCCAGCCACCTGA

AGTCCAAAAAGGGTCAGTCTACCTCCCGCCATAAAAAACTCATGTTCAAGACAG AAGGGCCTGACTCAGACTG
A172
ACTTCCTGAAAACAACGTTCTGTCCCCCTTGCCGTCCCAAGCAATGGATGATTTG ATGCTGTCCCCGGACGATATTGAACAATGGTTCACTGAAGACCCAGGTCCAGAT GAAGCTCCCAGAATGCCAGAGGCTGCTCCCC**G/C**CGTGGCCCCTGCACCAGCAGC TCCTACACCGGCGGCCCCTGCACCAGCCCCCTCCTGGCCCCTGTCATCTTCTGTC CCTTCCCAGAAAACCTACCAGGGCAGCTACGGTTTCCGTCTGGGCTTCTTGCATT CTGGGACAGCCAAGTCTGTGACTTGCACGTACTCCCCTGCCCTCAACAAGATGTT TTGCCAACTGGCCAAGACCTGCCCTGTGCAGCTGTGGGTTGATTCCACACCCCCG CCCGGCACCCGCGTCCGCGCCATGGCCATCTACAAGCAGTCACAGCACATGACG GAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGCTCAGATAGCGATGGTCTG GCCCCTCCTCAGCATCTTATCCGAGTGGAAGGAAATTTGCGTGTGGAGTATTTGG ATGACAGAAACACTTTTCGACATAGTGTGGTGGTGCCCTATGAGCCGCCTGAGTT GGCTCTGACTGTACCACCATCCACTACAACTACATGTGTAACAGTTCCTGCATGG GCGGCATGAACCGGAGGCCCATCCTCACCATCATCACACTGGAAGACTCCAGTG GTAATCTACTGGGACGGAACAGCTTTGAGGTGCGTGTTTGTGCCTGTCCTGGGAG AGACCGGCGCACAGAGGAAGAGAATCTCCGCAAGAAAGGGGAGCCTCACCACG AGCTGCCCCCAGGGAGCACTAAGCGAGCACTGCCCAACAACACCAGCTCCTCTC CCCAGCCAAAGAAGAAACCACTGGATGGAGAATATTTCACCCTTCAGATCCGTG GGCGTGAGCGCTTCGAGATGTTCCGAGAGCTGAATGAGGCCTTGGAACTCAAGG ATGCCCAGGCTGGGAAGGAGCCAGGGGGGAGCAGGGCTCACTCCAGCCACCTG AAGTCCAAAAAGGGTCAGTCTACCTCCCGCCATAAAAAACTCATGTTCAAGACA GAAGGGCCTGACTCAGACTG