Effects of Rosmarinic Acid on Protein Expression and Glycosylation of Glioblastoma Multiforme U87 Cell Lines in Comparison with Temozolomide

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This thesis is dedicated to my parents for their unconditional support and encouragement and my brother for his love and affection.

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List of Abbreviations

2DE	Two-Dimensional Electrophoresis
ALA	Alanine
APS	Amonium Per Sulphate
ARG	Asparagine
ASP	Aspartic Acid
cGMP	Cyclic Guanosine Monophosphate
CHAPS	Cholamidopropyl Dimethylammonio Propanesulfonate
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
EGFR	Epidermal Growth Factor Receptor
GLU	Glutamic Acid
GLY	Glycine
HNSCC	Head And Neck Squamous Cell Carcinomas
HSP-27	Heat Shock Protein 27
IEF	Iso-Electric Focusing
IL-17A	Interleukin 17 A
IPG	Immobilized Ph Gradient
iTRAQ	Isobaric Tags for Relative and Absolute Quantitation
LEU	Leucine
LYS	Lysine
MALDI TOF MS	Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry
MEF	Mouse Embryonic Fibroblasts
MET	Methionine
MG	Malignant Glioma
MTT	Dimethylthiazol-2
PBS	Phosphate Buffer Saline
PDB	Protein Data Bank
pI	Iso-Electric Point
PKG	Cyclic GMP–Dependent Protein Kinase
PMSF	Phenylmethylsulfonyl Fluoride

PRO	Proline
RA	Rosmarinic Acid
RMSD	Root Mean Square Deviation
ROS	Reactive Oxygen Specie
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SER	Serine
STRING	Search Tool for The Retrieval of Interacting Genes/Proteins
TCGA	The Cancer Genomic Atlas
TEMED	Tetramethylethylenediamine
TMZ	Temozolomide
TNF-a	Tumor Necrosis Factor Alpha
TRP	Tryptophan
TYR	Tyrosine
VAL	Valine
VEGF	Vascular Endothelial Growth Factor
GBM	Glioblastoma Multiforme

Abstract

Glioblastoma multiforme (GBM) is a tumor of glial origin and is most malignant, aggressive, and prevalent with highest mortality rate in adult brain cancer. The clinical treatment available is surgical resection of tumor followed with standard drug Temozolomide (TMZ). The current standard treatment has major limitations with 50% of patients' resistance to TMZ and average survival of 15 months after diagnosis. This underscores the need of investigating novel potential drugs and their targets in GBM. Rosmarinic acid (RA), natural constituent of Lamiaceae plants have reported neuropharmacological and anti-cancerous properties.

This study seeks to investigate the binding and interacting potential of rosmarinic acid with aberrant expressing proteins in GBM. This study evaluates the molecular interaction of rosmarinic acid to following target protein: Hsp27, EGFR, TNF-α, Annexin A2, IL17A, galectin-1 as protein-ligand interactions play a vital role in drug design. Automated docking studies were performed utilizing Autodock4 to provide useful insights into rosmarinic acid bindings to GBM potential targets. The results show encouraging therapeutic potential of RA against targeted proteins with strongest interaction and binding affinity to HSP-27 and TNF-a out of the selected proteins. The docking comparison of RA with standard drug Temozolomide indicated binding interactions of TMZ with selected targeted proteins. RA formed more stable conformation with targeted proteins compared to TMZ on the basis of binding energy and protein ligand orientation.

Uncharacteristic glycosylation has been associated with tumorigenesis and metastasis mechanisms. Sialic acid sugars attached to glycoproteins and glycolipids upregulation is a hallmark feature of tumor cell. Glycosylation can also be a potential diagnostic marker as well as a putative aim for therapy in GBM. The study also predicted the glycosylation sites in the above targeted proteins employing NetN Glycan software. Whereas protein glycosylation pattern was also investigated in U87 malignant glioma (U87-MG) cell lines. Moreover,

differential protein expression between sensitive and resistant variants of U87-MG cell lines to standard drug TMZ were studied using 2D gel electrophoresis and SDS-PAGE. Protein spot identification and statistics were performed with the help of Delta2D software. A total of 10 protein spots were identified as differentially expressed. These findings may aid in understanding the potential role of these proteins and can serve as potential biomarkers and drug targets for GBM.

Introduction

Glioblastoma Multiforme (GBM) has continued to maintain its position as one of the deadly cancers with least survival time (Smoll et al., 2013). GBM being the most prevalent among other brain tumors mostly targets the cerebral hemisphere's subcortical white matter within cortico-temporal region of brain (Alabama, 2010).

Gliomas are the brain tumours of glial origin. Glial cells support and maintains the neurons in brain. Out of all brain tumours, 30% of cases accounts for Gliomas. (Anjum et al., 2017) Histologically they are classified as astrocytic, oligodendroglia, or ependymal cells depending upon their morphology based on hypothesized line of differentiation. According to WHO (world health organization) gliomas are classified as astrocytic tumors (Grade I-IV), oligodendrogliomas, ependymomas, and mixed gliomas (Anjum et al., 2017). Gliomas are graded based on their degree of malignancy from I to IV. Grade I: pilocytic astrocytoma, Grade II: diffuse astrocytoma, Grade III: anaplastic astrocytoma, and Grade IV: glioblastoma (GBM). Glioblastoma multiforme (GBM) is the most malignant, aggressive, and common with highest mortality rate in brain cancer. The intra-tumoral heterogeneity of GBM makes it challenging for recognizing and then validating the biomarkers and exigent in therapeutic research due to their refractory responses to available treatment options (Suvasini et al., 2011). Besides histological classification, molecular classification of GBM can be exploited for targeted drug discovery. There are four subtypes based on molecular profiling; 1) Classical: with chromosome 7 over expression and chromosome 10 under, and EGFR up regulation, 2) Mesenchymal: *NF* loss, reporting *CD44* and *MERTYK*, markers of epithelial-to-mesenchymal transition, tumour necrosis factor genes and NF-κB pathways upregulation. 3) Proneural: *PDGFRA* expression alteration and *IDH1* mutation. 4) Neural: shows neural markers *NEFL, SYT1*, *GABRA1*, and *SLC12A5*. Gliomas are clinically characterized

histologically and genetically by *IDH1* and *IDH2* mutations and the methylation status of MGMT promoter for determining treatment approaches (Shergalis et al., 2018) (Taylor et al., 2019).

Patients diagnosed with GBM exhibit different symptoms depending upon the tumor location and size in brain region. For example, vision and hearing problems arise when the tumor is in temporal lobes, personality changes could arise with tumor in frontal lobe. Multitude of varying symptoms are shown in patients including seizures, vomiting, headaches, nausea, blurred vision, and even impaired cognitive functions. Hence the tumor is diagnosed and located with the help of MRI scans and correct diagnosis can be done after the tissue analysis of tumor resection. Stereotactic biopsy can be performed based on tumor location. Allowing the surgeons to remove the malignant tumor vital for the survival of patients (Hottinger et al., 2014).

Etiology of GBM is still unknown. There are multiple factors because of which we are unable to pin-point certain factors causing the disease. Tumor heterogeneity, with tumors located on different parts of same organ (inter-tumor heterogeneity) and also cells within the same tumor (intra-tumor heterogeneity), is the hallmark of GBM pointing towards different genetic and molecular causes (Anjum et al., 2017). Several cancer inducing molecular pathway mutations, genetic abnormalities contribute to glioma genesis, which makes it challenging to untangle the molecular cause. 60% of cases originate de novo while lower grade malignant tumors give rise to 40 % of the cases (Kanderi et al., 2021).

The most accepted GBM causes include complete or loss or heterozygosity of chromosome 10, p16INK4a deletion, p14ARF and p53 mutation, RB1 methylation, and MGMT methylation. These are the mutations in DNA repair mechanism hence called mutator phenotype. P53 pathway is also reported to be disrupted in GBM. Amplification in EGFR expression on chromosome 7 (An et al., 2018).

Non-Ionizing electromagnetic radiation even from cell phone use, pesticides, head trauma which triggers gliosis are associated with glioblastoma causes. Exposure to ionizing radiation has been linked to glioblastoma development because patients treated with radio therapy for acute lymphoblast leukemia (ALL) have frequently diagnosed with GBM and its proliferation (Alabama, 2010).

GBM is known as adult brain cancer, reported in people after 45 years of age. It rarely effects children with only 8.8 % of tumors in children are GBM. Older the person, greater the chances of GBM development. The average age of GBM patients is 62. It effects both genders but primary GBM is reported more in males while secondary more in females (Anjum et al., 2017).

Glioblastoma being the most damaging tumor, with recurrent relapse and fatal prognosis the survival time of the patients are very trifling. The mean period is 8-15 months after diagnosis and 3-9 months in case of recurrent tumor growth. The long-term survival is reported in very less percentage of patients, 2.2% survive 3 years and total 5 years survival is only reported in 5% of the patients (Hanif et al., 2017). With the improvement in drugs, survival time is expected to be increased in near future. On account of low no of long-term survival group, the identification and research on long term survival group biomarkers becomes challenging. Younger patients, lower ki-67 values (protein associated with tumor proliferation and growth), hypermethylation of MGMT (O-6-methylguanine-DNA methyltransferase) are associated with increased survival time (Smoll et al., 2013).

The treatment of GBM is challenging considering the heterogeneity of the condition, one drug that might be showing good results on one patient might not work for another. Years of research and multimodal treatments including surgery, radiotherapy combined with chemotherapy still gives the average survival rate of 15 months.

Radio therapy still holds the conventional status to prevent the local relapse after surgery. The dose depends upon tumor volume and patients' conditions, for patients up to 70 years of age 60 fractionated radio therapy with 60 Gy in 2-Gy fractions and for older patients hypo fractionated radiation therapy 40 Gy in 2.66-Gy fractions is given with adjuvant chemo therapy (Cabrera et al., 2016).

After the approval of temozolomide in 2005 by FDA, it is given as the first line of drug after surgery and also as adjuvant therapy along with radiation therapy. It is an DNA alkylating agent, but its acquired resistance is the problem. TMZ works by targeting guanine on O^6 and N^7 positions. It causes the apoptosis of cancerous cells and prevents DNA replication. Given in both oral as well as injectables, it's given in 150-200mg/ m^2 of body mass index. In is given in cycles for 5 days after 28 days. Total 6 such cycles are given and then the condition is assessed. The dose determination is important since at low doses MGMT DNA repair enzyme has reduced action and higher dose can lead to resistance even in sensitive patients. 50% of patients are irresponsive to TMZ treatment. TMZ sensitive patients have methylated MGMT (methyl-guanine-methyltransferase) while hypomethylation have been reported in resistant patients. MGMT (DNA repair enzyme) play role in resistance through repairing N7 and O6 positions of guanine which temozolomide alkylated (Yi et al., 2018). Methylation of promoter region of MGMT can lead to silencing of the enzyme which is associated with increased survival time.

TMZ resistance is multifactorial one and therefore, genomic and proteomic profiling of patients could be useful clinically (Lee, 2016) (Anjum et al., 2017). Before the approval of TMZ Nitrosourea was the most prescribed drugs for gliomas but because of its toxicity against liver kidney and bone-marrow, it is avoided now (Brada et al., 2010).

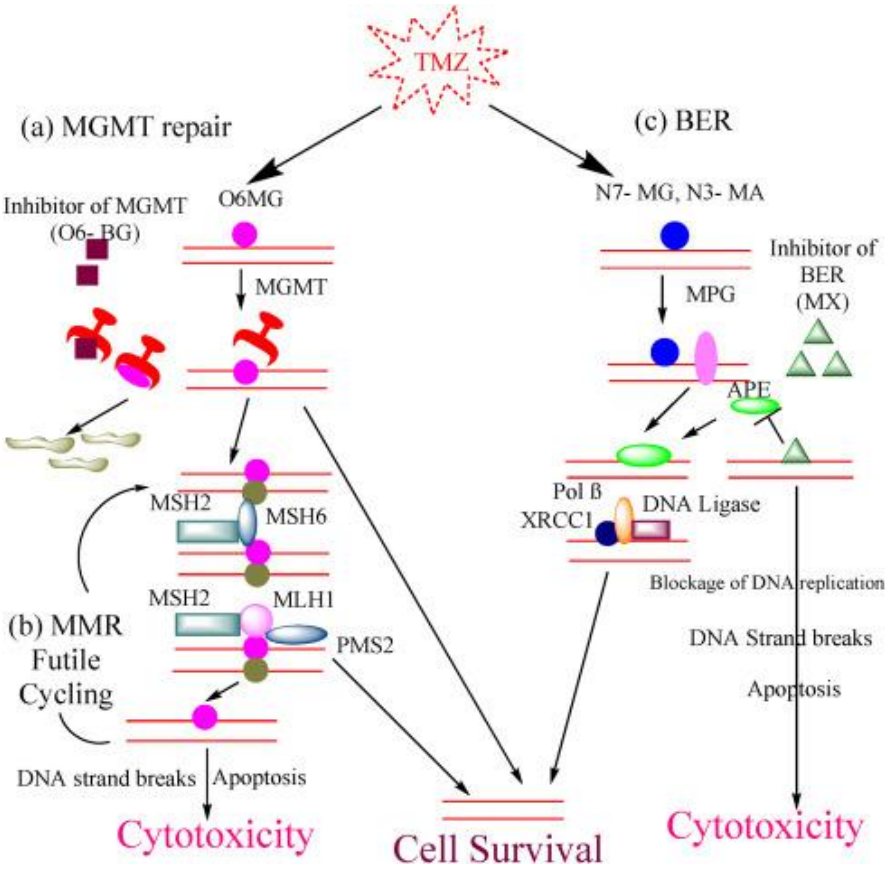


Figure 1. 1: Mechanism of action of TMZ against GBM (adapted from Anjum et al., 2017).

The unwanted side effects and resistance of the available drugs have intrigued the researchers to investigate the phenolic and carbonyl based natural active compounds. Literature data provides plethora of evidence regarding the neuropharmalogical application of Rosmarinus officialise (Ghasemzadeh et al., 2020). Rosmarinic acid (RA), an active compound of Rosmarinus officialise, is a phenolic carboxylic acid. Other Lamiaceae species plants also contain RA like lemon balm, oregano, peppermint , sage, and thyme. (Şengelen et al., 2018). RA has widely established properties as anti-inflammatory, antioxidant, and anti-microbial activities (Lamaison et al., 1991). RA has also reported as potent anti-cancer, anti-lipid peroxidation and apoptotic effect (Sharmila et al., 2012). In anti-cancerous properties, RA in a study has shown to supress cell proliferation and anti-oxidant effect at 80–130 μM

concentration and a prooxidant effect and cell death through necrosis was observed at 200 μM and higher concentrations (Ramanauskiene et al., 2016).

Glioblastoma tumor even after aggressive treatment with chemo and radio therapy, persist to be severely resistant to available treatment strategies and disease prognosis remain awfully low. Even with the advancements, continuous research and increase in available treatment, the GBM prognosis have not been improved considerably over past decades. These challenges have driven scientist to reevaluate the current treatment and explore new targets and compounds for GBM (Wu et al., 2021).

This study would focus on Rosmarinus officialises isolated constituent rosmarinic acid to find its interaction with GBM drug targets utilizing *in silico* docking tools. The glycosylation PTM prediction of the targeted proteins and its effect on GBM. To study the normal and aberrant cellular processes cancer cell lines are important model systems. U87MG (malignant glioma) is probably the most commonly used cell line for research on human glioma (Allen et al., 2016). U87 cell lines are TMZ sensitive (Taylor et al., 2019). Resistance in the sensitive cell lines can be introduced through gradual increased doze of TMZ administered to the cell lines. After successful development of resistance in u87 cell lines against the standard drug. This study aims to do comparative analysis of sensitive and resistant variant of the same parental U87 cell lines utilizing proteomic techniques including SDS-PAGE, 2D gel electrophoresis and PTM focusing on glycosylation. These studies could provide an insight to the cellular mechanism at protein level behind the reason of developing resistance.

Proteins are the functional units and final product of gene expression. *In silico* docking of selected targeted proteins against our natural phenolic compound of interest Rosmarinic Acid has potential to identify novel drug targets in GBM as well as the potential of RA as a prospective candidate for GBM treatment (Shergalis et al., 2018). To identify proteins as therapeutic targets, post translational modifications are needed to be evaluated. Identified

PTMs are characteristically traced as disease markers as well as many are targeted for specific drug therapies. (Karve et al., 2011).

Uncharacteristic glycosylation has been correlated with tumorigenesis and metastasis mechanisms. Sialic acid sugars linked to glycoproteins and glycolipids upregulation is a hallmark feature of tumour cell. Glycosylation can also be a potential diagnostic marker as well as a putative aim for therapy in GBM (Veillon et al., 2018).

Literature Review

Glioblastoma, the grade IV cancer is challenging to treat even with the current advancements because of its genetic heterogeneity, blood brain barrier protection, immunosuppressive microenvironment, and high infiltration. Even the targeted therapy has not been success because of multiple factors driving the same tumor. Hence, it has been proposed that personalized target inhibition and combination of inhibitors targeting the aberrant expressed targets could show improved results. This also has limitations until now because of lack of effective therapies option available (Hottinger et al., 2014).

MicroRNA (miRNA) exogenous non-coding RNA, differential expression and their downstream pathway has been linked with GBM. Utilizing the in silico tools of Gene Expression Omnibus (GEO) database and The Cancer Genome Atlas (TCGA) database a miRNA -mRNA regulatory network and data on differential expression has been constructed (Lou et al., 2019). 10 up and 23 down regulated miRNA were reported on comparison with normal tissues. The up-stream transcription factors can regulate these mi-RNA so FunRich software was used to predict them. Potential target genes were predicted with miRNet database and then Cytoscape software to shortlist the HUB genes.it was found that cGMP-PKG signaling pathway was affected by the found down regulated genes and PI3K-Akt signaling pathway linked with the identified up-regulated genes. These pathways are linked with tumors and glioma progression. The identified genes e.g., EGFR and VEGFA controls glioma growth, MYC promotes glycolysis and spread of GBM. CD44 has role in GBM malignancy (Lou et al., 2019).

EGFR is transmembrane tyrosine kinase receptor, and 57.4% of primary GBM cases report increase in EGFR gene expression and hence upregulated levels of EGFR are associated with GBM. RA have been reported as an inhibitor to epidermal growth factor (EGF) (An et al., 2018).

RA role as inhibitor of epidermal growth factor receptor (EGFR) triggers head and neck squamous cell carcinoma (HNSCC) signalling and initiating ROS mediated mechanism. RA has reported to cause the inhibition of cancerous cell viability, proliferation, and cellular production of ROS in HNSCC cell lines. Additionally, RA at tyrosine residues 992 and 845 of the EGFR, inhibited EGF-induced phosphorylation which results in the downregulation of the phosphatidylinositol 3-kinase Akt (PI3K/Akt) and mitogen-activated protein kinase ERK (MAPK/ ERK) pathways (Tumur et al., 2015). EGFR upregulation in GBM and its reported inhibitory effect with RA in cancer was the reason to study its docking with RA.

Hsp27 is a molecular chaperone. The resistance of glioma cells to apoptosis is because of Hsp27 (Şengelen & Önay-Uçar, 2018). Different brain tumours such as gliomas, astrocytomas and oligodendrogliomas show upregulated Hsp27 (Zhang et al. 2003). Upregulation of Hsp27 is associated with cancer cell proliferation, differentiation, metastasis and prevent apoptosis. Drug resistance and cancer cell survival in lethal conditions are also linked with Hsp27. The studies suggest carcinogenesis stimulation by Hsp27. Down-regulation/inhibition of Hsp27 has been done in first round of studies by administering its chemical inhibitors or anti sense oligonucleotide in cancer therapy. The results showed considerable tumour volume reduction compared to traditional chemotherapeutic agents which only modestly reduced volume of tumour in in vivo studies (Vidyasagar et al., 2012). So, this could be targeted and its interaction with RA was checked through docking.

The upregulation of IL17A in GBM is the most prominent compared to other GBM biomarkers. Autocrine and paracrine growth factors (VEGF) and immune cells are activated in Glioma cells by IL17AR and IL17A interaction. This promotes the vascularity and angiogenesis by glioma-infiltrating immune cells at the inflammation site which aggravates the GBM (Hu et al., 2013).

A lectin family protein, Galectin-1 has galactoside affinity and carbohydrate-recognizing domain. It is a ubiquitous protein involving in no of biological functions like cell proliferation, cell adhesion, tumour metastasis, immunoregulatory effects and apoptosis, have multiple receptors. Galectin-1 up regulation is reported in multiple cancers including head and neck squamous cell carcinomas, endometrial and prostate. Galectin-1 expression is also associated in all human glioma types and is associated with malignancy and poor prognosis (Camby et al. 2006). It is considered to be playing role in suppression of antitumor resposes, immune tolerance in gliomas, and diminution of chronic inflammation. Galectin link between innate and adaptive responses could be taken as a novel regulatory checkpoint. Its up regulation in autoimmune inflammation and broad immunoregulatory effects validation in multiple study models of cancer makes it a biomarker of malignant tumour progression. Hence its targeted inhibition is of interest for therapeutic strategy (Mendez-Huergo et al., 2019). Its interaction with RA has been observed.

Annexin A2 is a calcium-binding cytoskeletal protein. Multiple studies have reported its expression correlation with metastasis, tumor aggression and decreased glioma survival rate making it a considerable drug target. It has functional role in plasminogen to plasmin (serine protease) conversion which stimulates metalloproteinases and degrades the extracellular matrix which results in increased cell metastasis. Its continual upregulation in GBM makes it a promising drug target and hence its interaction with RA were studied (Shergalis et al., 2018).

TNF-α stimulate cell proliferation pathway but its inhibition can cause apoptosis of carcinoma cells. RA has reported this result by possible interaction with TNF-α on human leukemia U937 cells. It happens by activation of NF-κB and ROS which makes cancer cells vulnerable to induced apoptosis. Moreover, RA treatment has shown increase caspases activation through TNF-α (Moon et al. 2010) (Kim et al. 2010). Although TNFα's role in cell

survival and apoptosis is defined but its function in cell survival and death maintenance remain vague. RA, through TNFα pathway effects the pro and anti- apoptotic maintenance of cell (Van Horssen et al. 2006).

Molecular level understanding of malignant brain tumors was investigated on U87 and U343 malignant human glioma cell lines. Their motility, invasiveness and viability were assessed in invitro experiments and found to be different. 2D gel electrophoresis was performed to find significant alteration between the cell lines in order to have an understanding behind the differences and glioma invasion. 9 different spots identified from the 2D gel with Phoretix 2D advanced software selected and subjected to MALDI-TOFMS to identify proteins. Cathepsin D was the protein which was identified to play role in glioma invasion. Further investigation of Cathepsin D role in glioma should be investigated (Pei et al., 2014).

Cell lines are ideal experimental models and U251 and U87 cell lines are most preferred for GBM studies. Difference in these cell line's protein expression profile utilizing 2d gel electrophoresis and iTRAQ was studied to find the distinction at molecular level. These both cell lines derived from glioma tumor show difference in in migration and invasion capability. 507 proteins were found which showed two times difference between the two. Nine of these were further validated with quantitative PCR. In U251 cell lines, the upregulated proteins were mostly extracellular and linked with metabolism of nicotinamide nucleotides and carboxylic acid, adenyl nucleotide binding and cellular distribution. While in U87 cell lines the over expressed genes were mostly intracellular and associated with mRNA processing and RNA splicing, poly(A) RNA binding. Moreover, U87 cell lines are more malignant and invasive in nature. The results from this study also concludes that purine metabolism is more lethal to U251 if targeted compared to U87 (Qi & Liu, 2016) .

A phase 3 trial conducted by National cancer institute of Canada and European organization of research of cancer was performed with random 573 study subjects with GBM to whom

radio therapy was given at 60 Gy compared with the group which was also given adjuvant Temozolomide. The purpose was to find the rate of improvement in survival with adjuvant therapy and it was found 12.1 to 14.6 months were on average increased with TMZ with overall increased in long term survival of 5 years from 1.9% to 9.8% ($p<0.0001$). It was concluded from meta-analysis that addition of TMZ following resection along with radio therapy improves survival (Cabrera et al., 2016).

High dose of etoposide, a drug for GBM, has toxic effects and it was checked on U87-MG whether the addition of Rosmarinus Officinalis, a natural compound, along with drug would have more efficacy. Neutral red and MTT assay were employed on cell lines with control MEF cell lines after the addition of drug. It was reported that Rosmarinus officinalis protects healthy cells against etoposide side effects while in U87 cytotoxicity of etoposide was not affected by Rosmarinus Officinalis (Ozdemir et al., 2018).

In Pakistan, 6.7 out of 1 lac people have central nervous system tumors and 51% of them are gliomas. Because of limited healthcare facilities and mostly glioma surgery offered by private hospitals which charge hefty amounts, rural and underprivileged population remain undiagnosed, untreated, or forced to leave treatment. For improving the current condition, neurosurgeons should be provided with better instrumental technology, more public sector hospitals equipped to deal with it, improve the follow-ups and post-surgery care rate, and research to investigate cost effective treatment options (Abdullah et al., 2019).

Glycosylation, one of the major post translational modifications, alters the protein physiochemical properties. It is the addition of carbohydrate moiety on protein side chain. N-linked glycosylation are the most important in eukaryotes. It occurs on sequon Asn/Xaa/Ser/Thr Asparginine , on condition when Xaa is not proline. Multiple artificial neural networks have been developed to predict the sites of these glycosylation in proteome (Gupta, 2002).

Molecular characterization of Huh-7 cell lines with major focus on glycosylation and bioactivity was researched. Densitometric analysis with SDS-PAGE with glycosylated protein indicated that 84% of extra cellular proteins were glycosylated and the target gene of study was more bioactive in non-glycosylated group (M. A. Khan et al., 2017).

Since glycosylation post translational modification has major functional roles like immune responses, intercellular signaling, inflammation and host-pathogen interactions. Their study in wet lab in laborious and challenging. Computational tools are designed to fill in the gap like SPRINT-Gly (Taherzadeh et al., 2019).

To improve the sensitivity, accuracy and specificity of glycosylation prediction was designed and the existing ones were compared based on these properties. These open source software has been a huge help in understanding post translational modification (Chien et al., 2020) (Pugalenthi et al., 2020).

Materials and Methods

1. In silico Study

1.1 Selection of Target Proteins

Identification and selection of potential target protein for the anticancer activity against GBM was done through literature review. The functional aspect of the protein and their associated pathways were identified utilizing online databases and resources including STRING 8.3 (string-db.org), Reactome (Reactome.org), UniProt (www.uniprot.org).

1.2 Protein Structure

3D structure of the selected proteins was retrieved from open online source, protein data bank (https://www.rcsb.org/CSB PDB) in PDB format. The protein structured was prepared for docking by removing water molecules and other unnecessary ligands utilizing the BIOVIA Discovery studio 2021 software.

1.3 Ligand Structure

The ligand, rosmarinic acid structure and standard drug temozolomide were obtained from PubChem (pubchem.ncbi.nlm.nih.gov/) database and saved after conversion in PDB format. SwissADME online software (http://www.swissadme.ch/), admetSAR (http://lmmd.ecust.edu.cn/admetsar2) and SCFBIO online software (scfbio-iitd.res.in) was utilized to check the drug-likeness of rosmarinic acid.

1.4 Molecular Docking

After collecting the structures, docking was performed on a software AutoDock4. It predicts interactions and preferred orientation of ligand on protein when bound to each other to form a stable complex. Knowledge of the preferred orientation can be used to predict the strength of

association or binding affinity between two molecules. AutoDock4 is based on Lamarckian Genetic Algorithm and empirical free energy scoring function (Rizvi et al., 2013). Binding pockets for the selected targeted proteins were identified with the help of online tool DoGSiteScorer (https://proteins.plus/). On uploading the protein 3D structure on the site and applying the DoDSiteScorer, the grid axes are given with durability scores. The binding pocket with highest scores nearest to 1 was selected. The grid coordinates of that pocket were set in autodock4 parameters to differentiate between active and random sites (Volkamer et al., 2012). Both proteins and ligands were converted into pdbqt files. Protein and ligand rigid conformation was selected during docking. The results are generated with 20 poses and the one with least binding energy was selected. The smaller the value of binding energy, the more is the ligand protein interaction and the more stable the conformation is. Hence, the more negative value of binding energy gives the best and most stable conformation of ligand interaction. The one best pose was chosen with the lowest binding energy.

1.5 Docking Analysis

The best pose interactions were studied in BIOVIA Discovery Studio, where the interacting residues of the active sites and bonds formed were analysed.

1.6 Glycosylation Prediction

The selected protein targets were also checked for glycoproteins prediction utilizing the online NetNGlyc - 1.0 software (https://www. cbs.dtu.dk/services/NetNGlyc/). The FASTA sequence of the proteins were retrieved from UniProt (https://www.uniprot.org/). The sequences were checked for glycosylation on NetNGlyc.

2. In Vitro Study

2.1 U87 cell lines

U87 cell lines both variants sensitive U87 and resistant R-U87 culture were maintained in DMEM (Gibco by life technologies catalogue# 31800-022) supplemented with 10% fetal bovine serum (gibco by life technology catalogue # 16050) and 1% penicillin streptomycin (Prenstrep Gibco by lifesciences technologies catalogue# 00580) to avoid contamination. The Culture were maintained at 37 $^{\circ}$C and 5% CO_2 in humidified incubator (Malacrida et al., 2020). For protein extraction, cell lines were passaged until an approximate 80% confluence was reached (Hong et al., 2012).

2.2 Protein Extraction

U87 cell lines grown to 80% confluency were taken and trypsinization of the cells were done. The cell count was estimated and according to the cell count 7 × 10^6 cells were taken. Cells were centrifuged and washed with 1x phosphate buffer saline (PBS) twice and were pelleted down. For approximately 7 × 10^6 cells, 500 ul lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 10 mM phenyl methyl sulfonyl fluoride (PMSF), 1% Dithiothreitol (DTT)), was added. After the addition of lysis buffer, cells were homogenized with UP400S Ultrasonic Processor (Hielscher Ultrasound Technology, USA) followed by incubation at 4°C for one hour with vortexing in intervals. Homogenized cells were centrifuged at 14000 rpm at 4 °C for 20 min. The supernatant was carefully taken in microcentrifuge tube and was stored at -80°C until further use (Pei et al., 2014).

2.3 Bradford's Protein Quantification Assay

The protein lysate was quantified with Bradford’s assay (Bradford, 1976) to calculate the concentration of protein in mg/ml. For standard curve, stock bovine serum albumin (1mg/ml)

was used to make serial dilutions in distilled water. Dilution of conc. range (0,0.05,0.10,0.20,0.40,0.60,0.80,1.00) was prepared with double distilled water to a final volume of 20 ul. Bradford's reagent (1ml) was added in standard dilutions and extracted protein lysate (1:20). After vortexing the samples were incubated in dark for 10 min, at room temperature. The absorbance was measured at 595 nm using visible spectrophotometer ((SP-300, OPTIMA, USA). Readings were taken for each sample (prepared in duplicates). The standard curve was generated by plotting the mean values of the absorption on y axis and known protein conc on x axis. This graph was used to calculate the concentration of the protein lysate extracted from cell lines.

2.4 Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Extracted and quantified protein lysate from U87 cell lines was then subjected to SDS-PAGE for the separation of proteins on the basis of their molecular mass. Sodium dodecyl sulphate (SDS) gives uniform charge to protein which is then separated on polyacrylamide gel electrophoresis in which acrylamide and bis acrylamide form cross linked gel matrix in the presence of APS and TEMED enzymes. Mini Protean Tetra Cell, (BioRad, USA) was set up. The glass plates were clean, assembled and checked for leakages. Resolving gel (10%) was first poured in glass plates carefully to the level leaving enough space for stacking gel. Ice cold isopropanol was added at the top to help level the gel surface and the gel was allowed to polymerize. Stacking gel (4%) was prepared and poured carefully to avoid bubbles formation, and comb was inserted.

2.4.1 Protein Sample Preparation

The quantified protein sample (50 ug) was loaded into the wells for SDS-PAGE. The sample was diluted (1:1) in sample diluting buffer (0.125 M Tris-HCI pH 6.8; 20 % Glycerol; 4% SDS, 2% Mercaptoethanol, bromophenol blue) and subjected to boiling at 95°C for 5 min.

The samples were then allowed to cool until it reaches room temperature and were loaded in gel along with protein page ruler (thermo-scientific pre-stained page ruler, catalogue# 26616).

2.4.2 Electrophoresis

The Mini Protean Tetra Cell, after loading the samples in gel was filled with electrode tank buffer (0.025M Tris pH 8.8, 0.192M glycine, 0.1% SDS). The electrophoresis was carried out at 100V until the gel was run completely. Gel running was tracked with tracking dye (bromophenol blue) present in sample diluting buffer. After electrophoresis, the gel was carefully placed in container filled with Coomassie staining solution (250ml propanol, 150ml glacial acetic acid, 0.25 Coomassie brilliant blue). The gel was allowed to stain over-night and later de-stained with detaining solution (20% ethanol, 10% acetic acid) on shaker and washed multiple times until the excess stain is removed and protein bands are visible.

2.4.3 Image analysis

The images of gel were taken on ChemiDoc (ChemiDoc™ MP System #1708280) under white light. Image Lab™ software (BioRad, USA) was used for quantification and molecular weight calculation of the protein bands. The relative quantity of each band was assessed to calculate the differential expression of proteins.

2.5 Protein Glycosylation

Protein samples were separated with SDS-PAGE following the protocol described in section 2.4. Positive and negative controls of glycoprotein stain were loaded along with samples. After electrophoresis was completed, the gel was stained using Glycoprotein Staining Kit (Thermo Scientific™ Pierce™, USA, Catalog number# 24562). The gel was first fixed in 50% methanol with constant shaking for 30 min and then immersed in 3% acetic acid with constant shaking for 10 min (twice). After fixation the gel was treated with oxidizing solution

for 30 min in dark. The gel was then washed with 3% acetic acid twice for 5 min and later transferred in glycoprotein stain and kept in dark on shaker for 30 min. The gel was placed in reducing solution for 15 min and then 3% acetic acid was added to stop the reaction. Imaging of gel was done on ChemiDoc (ChemiDoc™ MP System #1708280, BioRad, USA) after washing with distilled water.

2.6 Two-Dimensional Polyacrylamide Gel Electrophoresis (2D PAGE)

2DE separates protein complex on two properties. First dimension on the basis of their iso-electric point by iso-electric focusing and second dimension on the basis of their molecular weight by SDS-PAGE.

2.6.1 Rehydration of Strips

isoelectric focusing (IEF) were performed using IPG strips (ReadyStrip™ IPG Strips #1632000) of 7 cm with pH gradient range 3–10. 200ug of protein sample from U87 cell lines were taken and final volume was made up to 125 ul with rehydration buffer (ready prep2D starter kit Catalogue # 163-2105). The prepared sample was added in rehydration tray along the whole length of channel carefully to avoid bubble formation. With the help of forceps IPG strips cover was removed and strips were placed gel side down avoid trapping the bubbles and remove if any. After 1 Hour 1ml of mineral oil was added t avoid sample loss. The strips were left overnight for rehydration, without moving rehydration tray on working bench.

2.6.2 Iso-Electric Focusing:

Next day, the rehydrated strips were transferred on IEF focusing tray keeping the gel side up after removing the extra mineral oil on filter paper without letting the gel side touch it. Placing the +ve side on positive label anode of tray. Gel side up wicks were carefully placed at the ends of IPG strips after wetting them with nanopure water. Strips were covered again

with 500ul of mineral oil. +ve and -ve anodes were fixed at the focusing tray and then in in Protean i12 IEF system keeping the anode labels in check.

Protean IEF system was set as follow, after selecting the lanes in which strips were placed:

7cm Strip	Voltage	Time	Volt-Hours
Step 1	250	20 min	
Step 2	4000	2 hours	
Step 3	4000	-	14000 V-hours

After the completion of electrophoresis, the strips were placed in clean rehydrating tray gel side up and were stored at -80°C after wrapping the tray carefully until next step SDS-PAGE.

2.6.5 Equilibration of IPG strips

IPG strips were allowed to thaw before equilibration. Equilibration Buffer 1 was prepared in falcon in 10 ml equilibration buffer 1, 100mg DTT and very light sprinkle of bromophenol blue at the time of working was added. Strip was placed carefully in falcon and the cap was sealed with paraffin. It was kept in shaker for 30 minutes.

Equilibration buffer 2 was prepared by adding 400mg of iodoacetamide and very light sprinkle of bromophenol blue right before working. The strips were then transferred with the help of forceps from equilibration buffer 1 to 2 and again kept on shaker for 30 minutes.

After equilibration strips were dipped for a second in electrode tank buffer before fixing on SDS-PAGE gel.

2.6.6 SDS-PAGE

Mini Protean Tetra Cell, (BioRad, USA) was set up. The glass plates were clean, assembled and checked for leakages. Resolving gel (10%) was first poured in glass plates carefully to

the level leaving enough space of stacking gel, strip and agarose sealing. Add chilled isopropanol at the top to help level the gel surface. Allowed the gel to polymerize completely, it takes approximately 40 minutes. After polymerization prepare stacking gel (4%) was prepared and poured carefully and slowly to avoid bubbles formation. On the polymerized stacking gel, carefully place the IPG strip with gel side on front, the strip was fixed with thin spatula in the stacking gel slightly and 1ul of protein page ruler was poured on small piece of filter paper and was fixed in stacking gel as well on the positive side of the strip. The gel was then sealed with 0.5% agarose. The gel was then set to run at 100V until complete run.

2.7 Silver Staining

Pierce silver staining kit (catalogue# 24612) was utilized for staining. It is more sensitive and rapid than Coomassie. The gel was first washed with distilled water for 5 minutes twice. Then it was fixed in 30% ethanol: 10%acetic acid: 60% water for 15 minutes twice. After fixing, gel was washed in 10% ethanol of 5 minutes 2 times. Then sensitizer working solution was added for 1 minutes then washed for two times with water. Then gel was stained for 30 minutes. Then after washing gel for 20 seconds in water, the gel was developed in developer working solution approximately in 1-2 minutes until spots are visible. Over developing could lead to undesirable dark background. As soon as the spots are appeared the developing should be stopped with stop solution 5% acetic acid. The images of gel were taken on Chemi-dock (ChemiDoc™ MP System #1708280).

2.8 Image and Statistical Analysis

The 2DE gel images were analyzed with Delta 2D 4.8 software. Duplicate gels were grouped and then warped together to one master gel. The identified spots by the software and their quantitation based on normalized % volume was also checked by visualizing the gel by naked

eye. The differentially expressed protein spots were selected and their expression profile graphs were prepared on graph pad prism 9.

Results

1. In silico Studies

1.1 Docking of selected target proteins with Rosmarinic Acid

Aberrantly regulated proteins and protein biomarkers of GBM were selected through literature review and then their functional aspect and role in tumorigenesis was identified using online software string, uniport and reactome. The selected proteins are: Hsp27, EGFR, TNF-α, Annexin A2, IL17A, galectin-1. The 3D structures of selected proteins retrieved from Protein Data Bank in PDB format were visualized and prepared for docking in Discovery Studio Biovia.

The ligand for docking, our compound of interest for investigation of its interaction with GBM drug target was retrieved from PubChem Data Base in PDB format along with the standard first line drug given to GBM patients Temozolomide (TMZ).

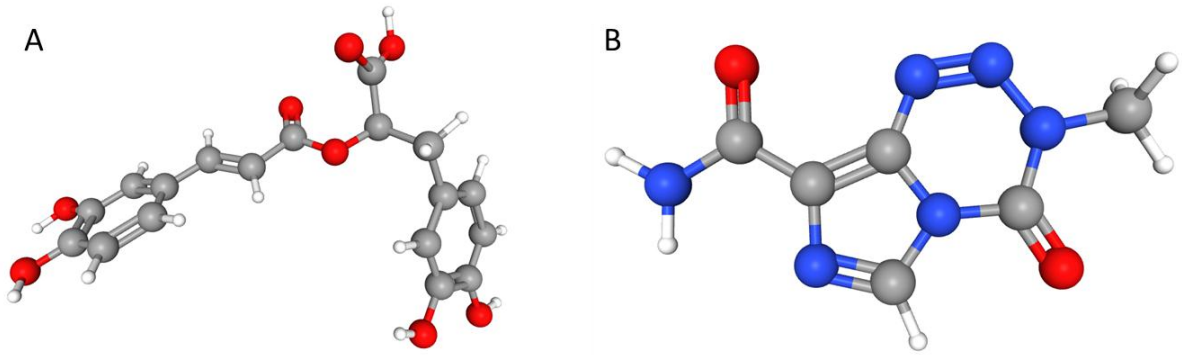


Figure 4.1: 3D structures of Temozolomide (TMZ Compound CID: 5394 (a) and Rosmarinic Acid (RA) Compound CID: 5281792 (b) obtained from PubChem.

The Drug-Likeness Prediction from SwissADME showed no violation to Lipinski rule for RA. Lipinski rule values for RA from SCFBio are listed in the table 4.1. The ADMET analysis gave absorption, distribution, metabolism, excretion, and toxicity prediction of RA. It revealed its absorption through blood brain barrier and intestine, non-AMES and carcinogenic toxicity. The *In-silico* drug likeliness analysis concludes to its potential as a drug.

LIPINSKI RULE ANALYSIS

MASS	<500 Daltons	360.00
HYDROGEN BOND DONOR	<5	5
HYDROGEN BOND ACCEPTORS	<10	5
LOGP	<5	1.761300
MOLAR REFRACTIVITY	40-130	89.796974

Table 4.1: Drug likeness prediction of RA with Lipinski Rule of 5.

AutoDock4 was employed to perform docking between the selected protein targets and ligand, the RA in comparison to standard drug TMZ. Both protein and ligands were considered as rigid structures. From the docking results, the pose with least binding energy and RMSD 0 were selected, which shows the highest affinity and stabilization of protein ligand complex. The inhibition constant (Ki) was also noted of the best selected pose, the calculated Ki from Auto dock 4 which could be used as a preliminary tool for screening inhibitors before executing experimental activity evaluation. Table 4.2 shows the binding affinities of the docked complex with RA and TMZ along with the inhibition constants.

The binding affinity of RA with the selected reported upregulated proteins of GBM were observed to be stronger than TMZ in all proteins. TNF-α showed the strongest affinity with -12.55 Kcal/Mol binding energy followed by IL17A and HSP27 with -11.84 and -11.43 Kcal/mol binding energy respectively. Annexin A2, EGFR and Galectin-1 showed -10.69, -9.46 and -8.97 Kcal/Mol of binding energies respectively. The inhibition constant Ki with RA also showed lesser quantity required for inhibition of the target proteins compared to TMZ. The Ki of all the docked complexes with RA were in nM (TNFa was in pM) while that of TMZ were in uM. The Ki value obtained also supports the hypothesis that RA not only shows stable interaction based on binding energy but also lesser Ki values which indicates concentration required to induce half maximum inhibition. Table 3.2 shows the Ki values along with binding affinities.

PROTEIN TARGETS	**PDB ID**	**LIGAND**	**BINDING ENERGY (KCAL/MOL)**	**INHIBITION CONSTANT (KI)**
IL17A	2VXS	RA	-11.84	2.10 nM
		TMZ	-5.8	51.99 μM
EGFR	1IVO	RA	-9.46	116.53 nM
		TMZ	-5.68	68.23 μM
HSP-27	6DV5	RA	-11.43	4.15 nM
		TMZ	-5.55	87.73 μM
TNF-A	7JRA	RA	-12.55	627.08pM
		TMZ	-7.34	4.18 μM
GALECTIN-1	1GZW	RA	-8.97	265.50nM
		TMZ	-6.16	30.53 μM
ANNEXIN A2	1W7B	RA	-10.69	14.56 nM
		TMZ	-5.27	138.11 μM

Table 4.2: The selected target proteins and their binding affinity with ligand RA and TMZ. Binding energy and inhibition constants of target protein with ligand RA and TMZ predicted by autodock4.

The best selected poses of ligands with the lowest binding energies were further analyzed in BIOVIA Discovery Studio. The interacting residues of the binding pockets along with the type of interactions and their bond lengths were analyzed Figure 4.2-4.7. All the docking complex generated exhibited conventional H bonds, hydrophobic and ionic interactions with amino acids in the binding pockets. H- bond is of high importance here as the ligand protein

complex is mainly stabilized by it. The Table 4.3 shows all the interaction amino acid at active sites, amino acids involved in H-bond formation and H-bond length. H- bond length 2.7 to 3.3 Å is generally observed so the bond distance below that is considered significant. All the protein ligand docked complex showed one or more hydrogen bonds with bond length below 3.3 Å. All protein targets showed more no of H-bond formation with RA compared to TMZ except EGFR which forms a H-Bond at SER (2.45 Å) with RA and forms three H-Bond at TYR C37 (3.29 Å) and ASP C46 (2.26 Å, 2.34 Å) with TMZ. The table 3.3 shows the complete list hydrogen bonds formed along with their lengths with RA and TMZ and it can be deduced that based on the H-Bond formation RA forms more stabilized structure with target proteins compared to TMZ. Nonetheless TMZ itself demonstrated well-established bonds both hydrogen and other hydrophobic interactions with all the target proteins. TNF α forms the most, 8 H-Bonds with the active sites residues with RA followed by IL17A with 5 H-bonds. Hence these proteins TNF α and IL17A showed the most stable conformation based on the binding affinities and H-bond interactions with active sites.

PROTEIN TARGETS	LIGAND	INTERACTING RESIDUES	CONVENTIONAL H- BOND INTERACTION	H- BOND LENGTH (A°)
IL17A	RA	TRP A44 SER A41 VAL A119 SER A118 TRP A67	SER A41 VAL A119 SER A118 TRP A67	2.11 2.35 2.37, 2.60 1.87
	TMZ	PRO A37 LEU A53 TRP A51 SER A 41 TRP A65 SER 118	TRP A65 SER 118	2.12 2.48, 2.29
EGFR	RA	MET A30 LYS C48 TRP C49 ASP C49 SER A26	SER A26	2.45
	TMZ	VAL C35 ASP A22 LEU A25 TRP C50 ARG A29 TYR C37 ASP C46	TYR C37 ASP C46	3.29 2.26, 2.34
HSP-27	RA	ASP A21 ARG A20 TYR A73 LEU A77	TYR A73 LEU A77	2.55, 2.00 2.98
	TMZ	GLY A13 PRO A159 TRP A22 TYR A23 ARG A20	ARG A20	2.82, 2.12
TNFA	RA	ARG A108 ALA A94 ASN A110 SER A223 GLY A224 GLN A225 VAL A226	ASN A110 SER A223 GLY A224 GLN A225 VAL A226 ALA A94	1.97 3.16 2.95 2.87 2.14, 1.87 2.139, 2.87
	TMZ	VAL A93 SER A223 ALA A109 ALA A94	ALA A94 ARG A108 GLU A222 GLY A224	3.24 2.80 1.96 2.90

		ARG A108 GLU A222 GLY A224		
GALECTIN-1	RA	LYS A127 GLU A22 GLY A124 GLY A82 GLU A22	GLY A124 GLY A82 GLU A22	2.00, 1.84 2.74, 2.70 3.27
	TMZ	PHE A126 ILE A128 LEU A4 LYS A127 GLY A 124	LEU A4 LYS A127 GLY A 124	2.84 2.73 1.95
ANNEXIN A2	RA	LEU A140 ARG A179 LEU A174 ILE A132 VAL A222 ALA A180	ILE A132 VAL A222 ALA A180	2.69 3.34 2.29
	TMZ	VAL A293 TYR A109 GLY A101 LYS A104 PRO A106 ARG 135 ARG A295	ARG A295	2.92

Table 4.3: The target proteins and their interactions with RA and TMZ observed on Image Lab BIOVIA. Active site interacting residues of target proteins with RA and TMZ of the best pose of AutoDock. Amino acid present at the binding sites of target protein that are involved in h-bond formation and their lengths observed within significant range.

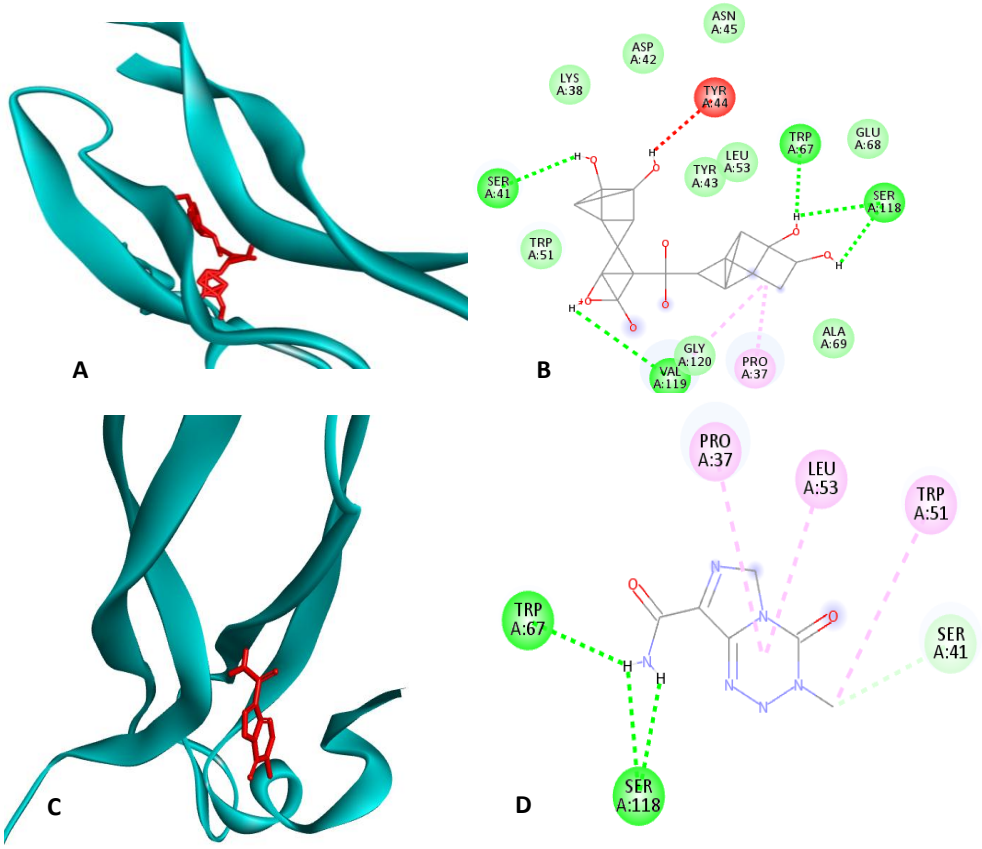


Figure 4.2: Molecular docking of IL17A with Rosmarinic acid and TMZ visualized by BIOVIA Discovery Studio. The Protein IL17A represented in blue ribbon model with red ligand. A. RA (red) best pose as predicted by Autodock4 with protein. B. 2D structure of RA representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond. C. TMZ (red) best pose as predicted by Autodock4 with protein. D. 2D structure of TMZ representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond.

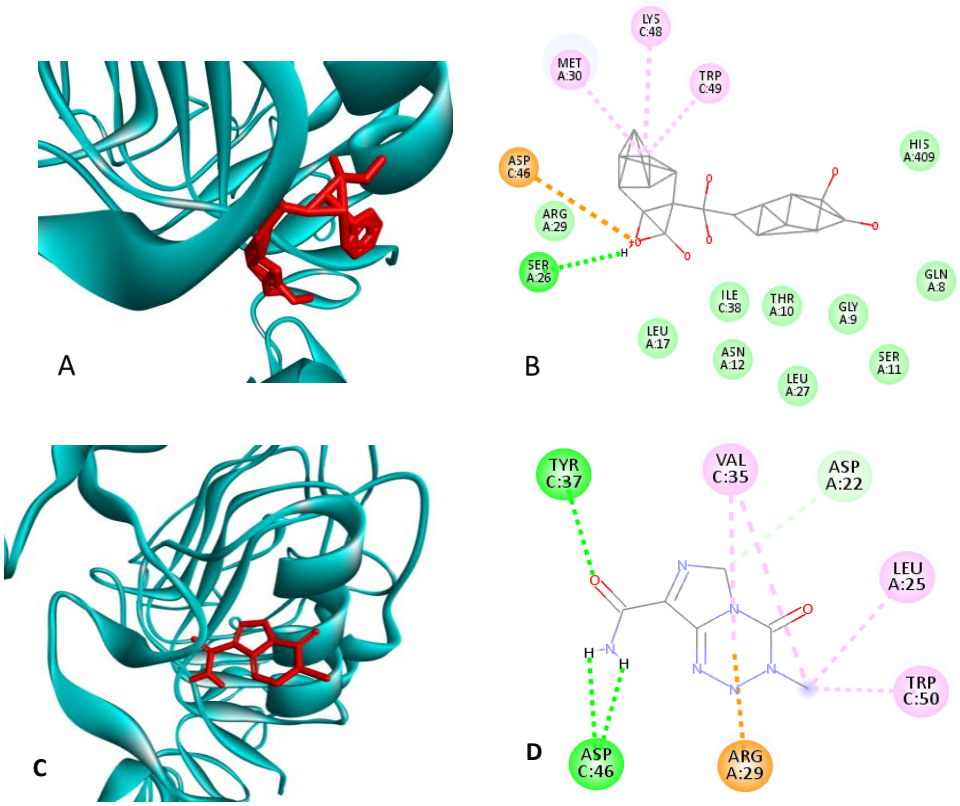


Figure 4.3: Molecular docking of EGFR with Rosmarinic acid and TMZ visualized by BIOVIA Discovery Studio. The Protein EGFRA represented in blue ribbon model with red ligand. A. RA (red) best pose as predicted by Autodock4 with protein. B. 2D structure of RA representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond. C. TMZ (red) best pose as predicted by Autodock4 with protein. D. 2D structure of TMZ representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond.

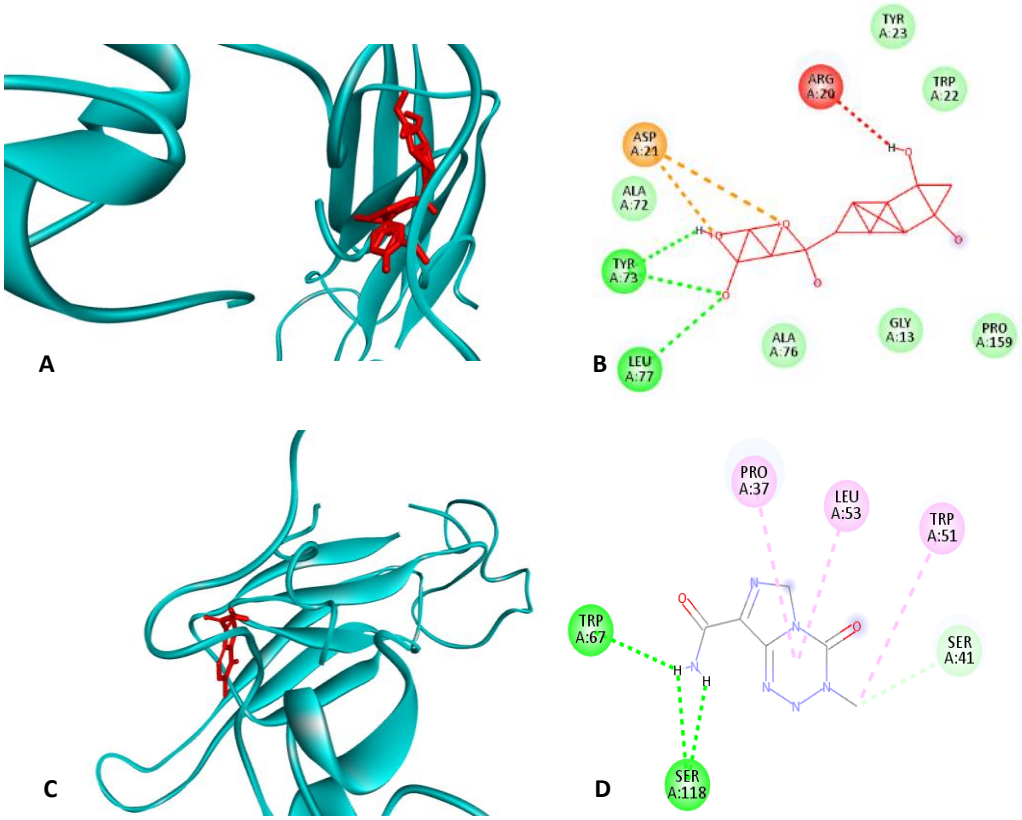


Figure 4.4: Molecular docking of HSP-27 with Rosmarinic acid and TMZ visualized by BIOVIA Discovery Studio. The Protein HSP-27 represented in blue ribbon model with red ligand. A. RA (red) best pose as predicted by Autodock4 with protein. B. 2D structure of RA representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond. C. TMZ (red) best pose as predicted by Autodock4 with protein. D. 2D structure of TMZ representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond.

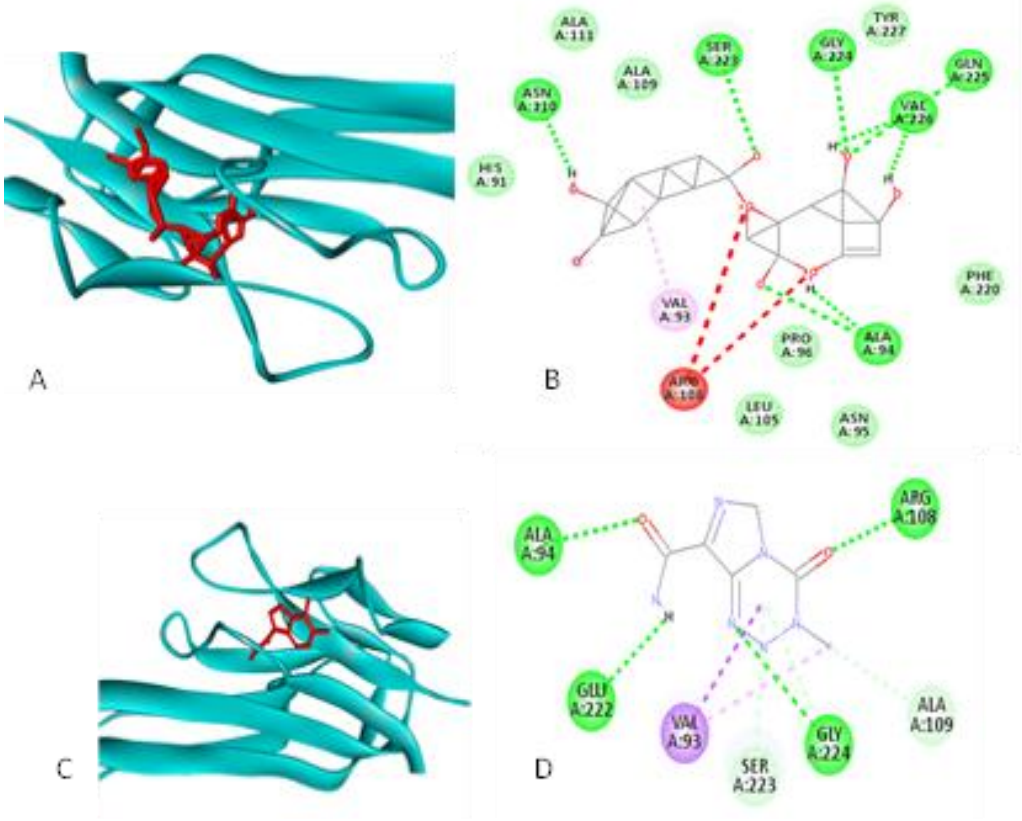


Figure 4.5: Molecular docking of TNFα with Rosmarinic acid and TMZ visualized by BIOVIA Discovery Studio. The Protein TNFα represented in blue ribbon model with red ligand. A. RA (red) best pose as predicted by Autodock4 with protein. B. 2D structure of RA representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond. C. TMZ (red) best pose as predicted by Autodock4 with protein. D. 2D structure of TMZ representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond.

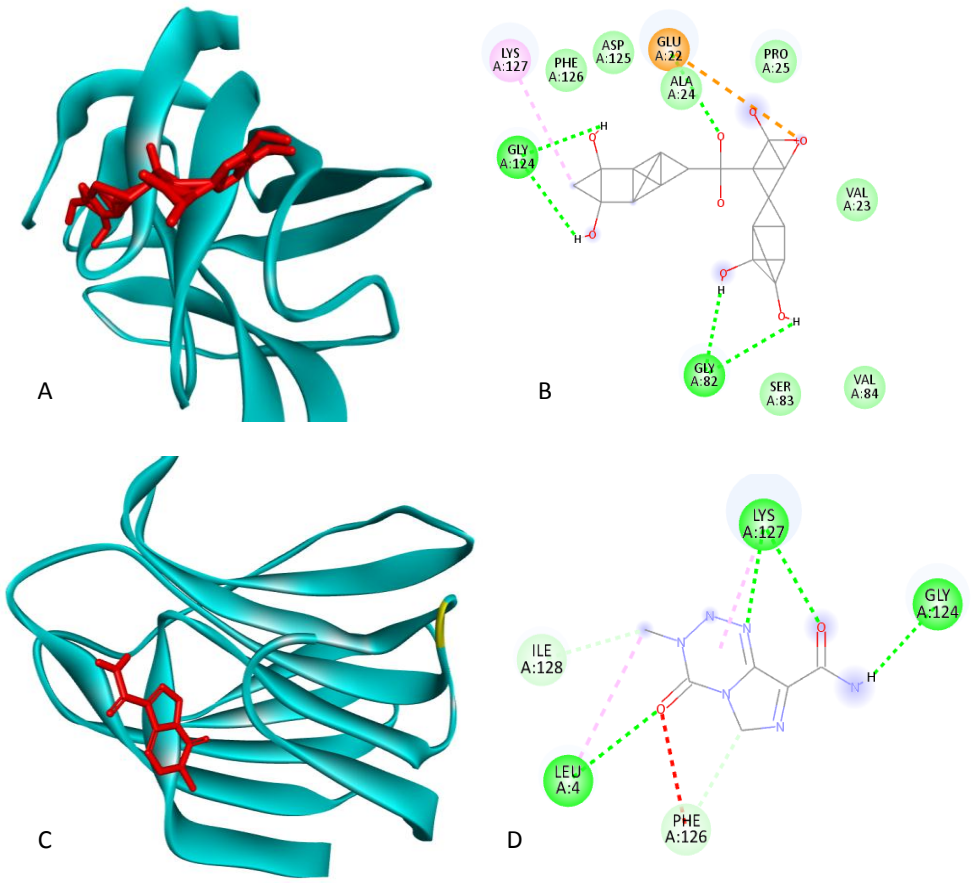


Figure 4.6: **Molecular docking of Galectin-1 with Rosmarinic acid and TMZ visualized by BIOVIA Discovery Studio.** The Protein Galectin-1 represented in blue ribbon model with red ligand. A. RA (red) best pose as predicted by Autodock4 with protein. B. 2D structure of RA representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond. C.TMZ (red) best pose as predicted by Autodock4 with protein. D. 2D structure of TMZ representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond.

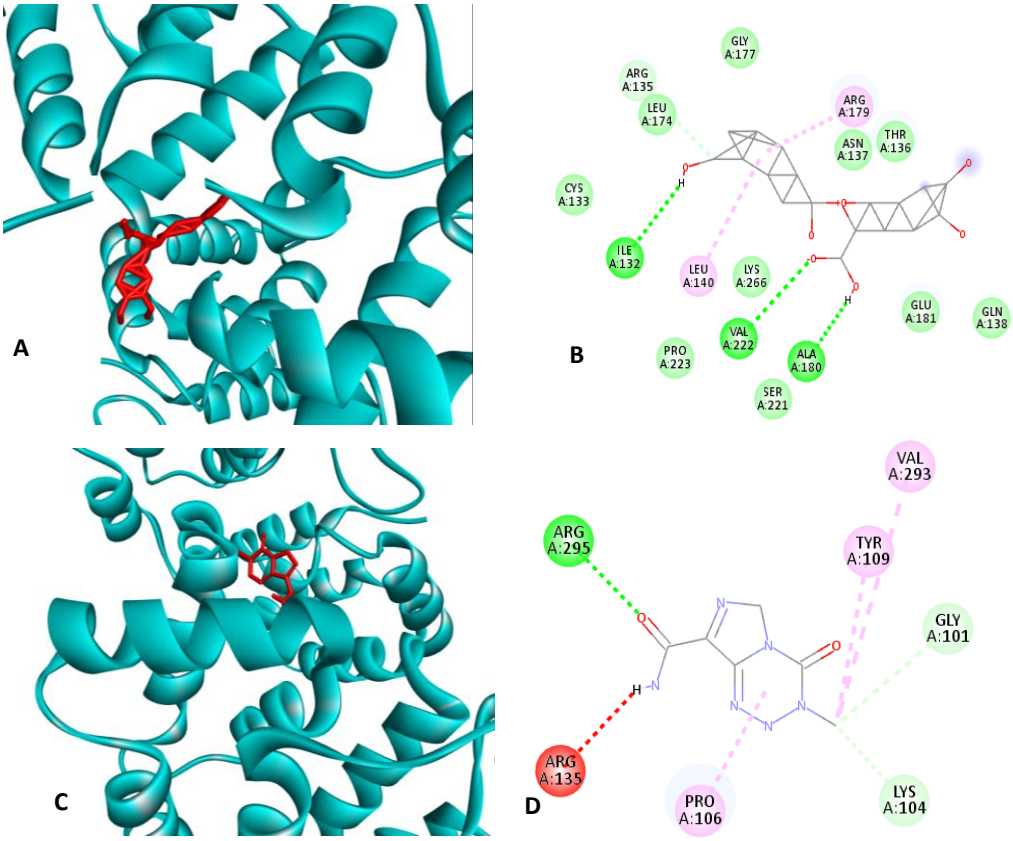


Figure 4.7: Molecular docking of Annexin A2 with Rosmarinic acid and TMZ visualized by BIOVIA Discovery Studio. The Protein Annexin A2 represented in blue ribbon model with red ligand. A. RA (red) best pose as predicted by Autodock4 with protein. B. 2D structure of RA representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond. C. TMZ (red) best pose as predicted by Autodock4 with protein. D. 2D structure of TMZ representing interacting residues at the binding site of protein along with bonds formed. Green dotted line represents H-bond.

2. In Vitro Study

2.1 Protein Quantification

The protein extracted from U87 cell lines was subjected to Bradford assay to quantify the concentrations of the samples. Standard graph was plotted with known protein concentration of range 0.0,0.05,0.1,0.2,0.4,0.6,0.8 and 1 on y- axis. The mean optical densities of the proteins measured at 595 nm was plotted on y axis. The concentration of U87 extracted protein was calculated from this graph.

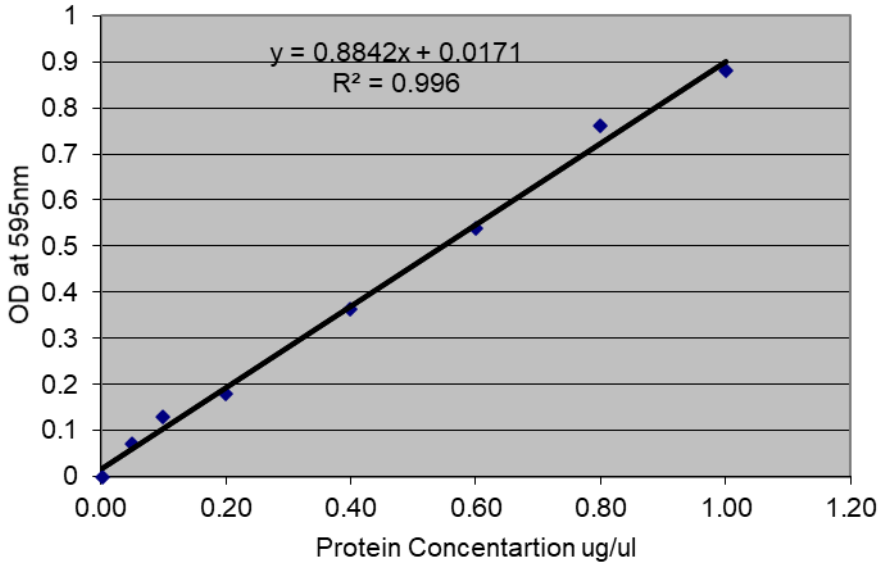


Figure 4.8: Protein quantification curve through Bradford assay. Bradford standard curve with known protein conc. on x-axis and OD obtained at 595 nm at y axis. The obtained linear regression value was 0.979 (R^2= 0.996).

2.2 Differential Protein Expression of U87-MG Variants

After iso-electric focusing and SDS-PAGE the gel was silver stained and visualized in Chemi-dock. Delta 2D 4.8 software was used for 2D gel analysis. Two replicates of both U87 Sensitive and U87 variants were warped and fused into one master gel with the software. The identified spots and their normalized % volume detected by software and were carefully verified by manual inspection. The figure 4.9 shows spots identified on fused gel in the software. The final selected spots with significant difference are represented in figure 4.10 and 4.11.

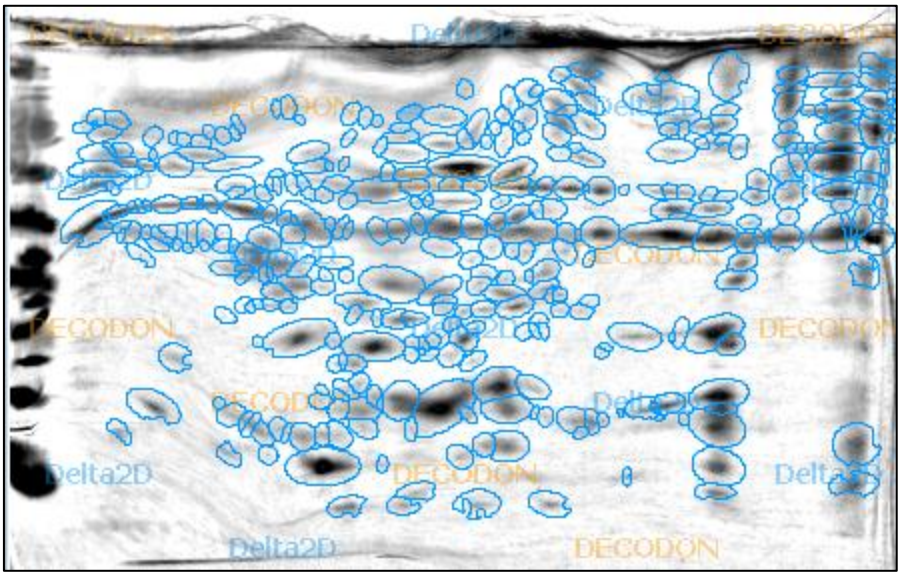


Figure 4.9: Representative gel with marked spots identified by Delta 2D software. The gel images (replicates) were warped and fused into single master gel. The spots were identified and quantified based on intensity.

The selected spots shown in the figure 4.10 and 4.11 was further subjected to quantitative analysis. The %age volume of each spot calculated by the Delta 2D software was subjected to statistical analysis with the help of graph pad prism 9. The graph of each spot is represented in figure 4.12 and 4.13. Statistics of paired t-test was applied for calculation of significance of differential expression ($p \geq 0.05$). Spots 1, 2, 5, 6 and 7 were found to be upregulated in resistant cell lines. Which means that the protein isolated at this spot is over expressed in the resistant U87-MG cell lines compared to sensitive U87-MG cell lines. However, the spots 3, 4, 8, 9 and 10 were found to be significantly less expressed in resistant compared to sensitive variant of U87-MG cell lines.

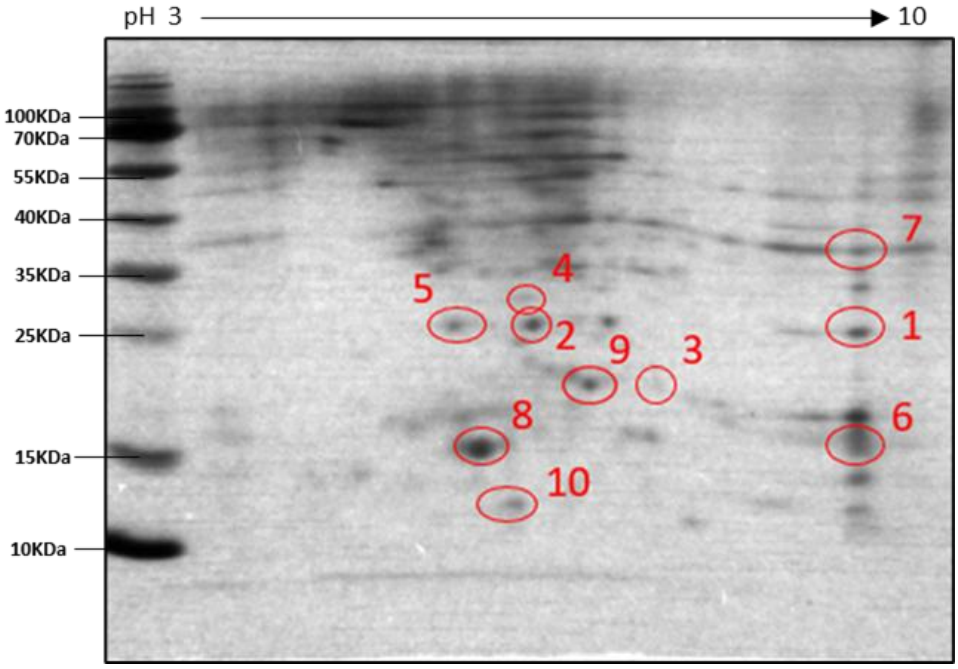


Figure 4.10*:* U87 Resistant cell lines protein separated by 2DE on non-linear pH range 3–10 IPG strip followed by SDS-PAGE and visualized by silver staining. The encircled spots indicate the differentially expressed proteins detected with Delta 2D software.

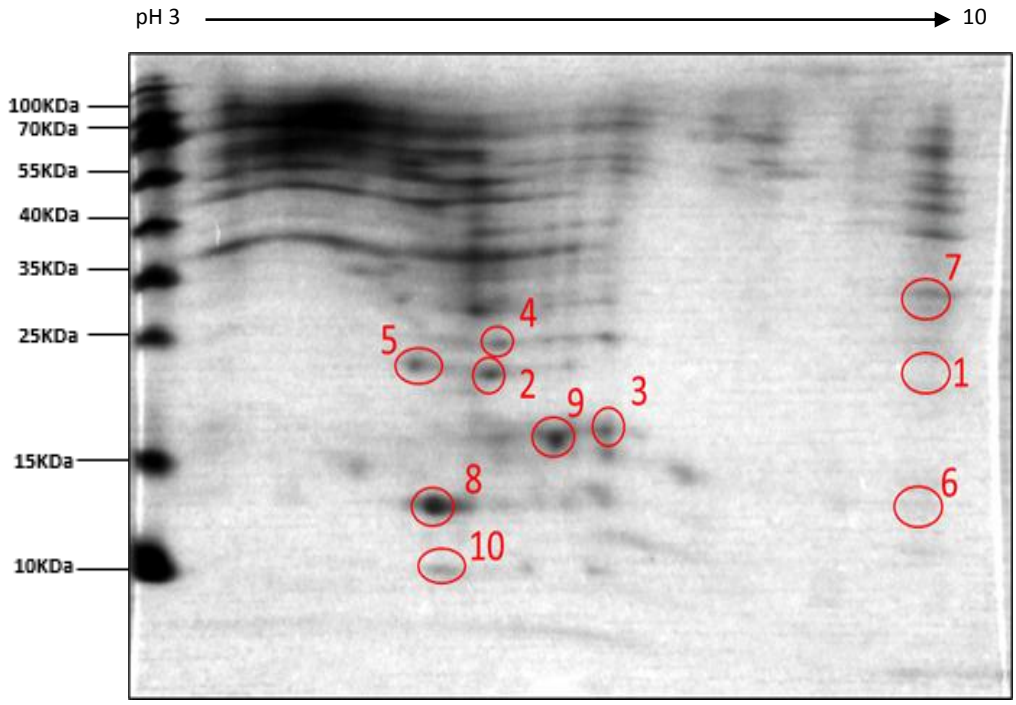


Figure 4.11: U87 sensitive cell lines protein separated by 2DE on non-linear pH range 3–10 IPG strip followed by SDS-PAGE and visualized by silver staining. The encircled spots indicate the differentially expressed proteins detected with Delta 2D software.

2.3 Differentially Expressed Protein in U87-MG Variants

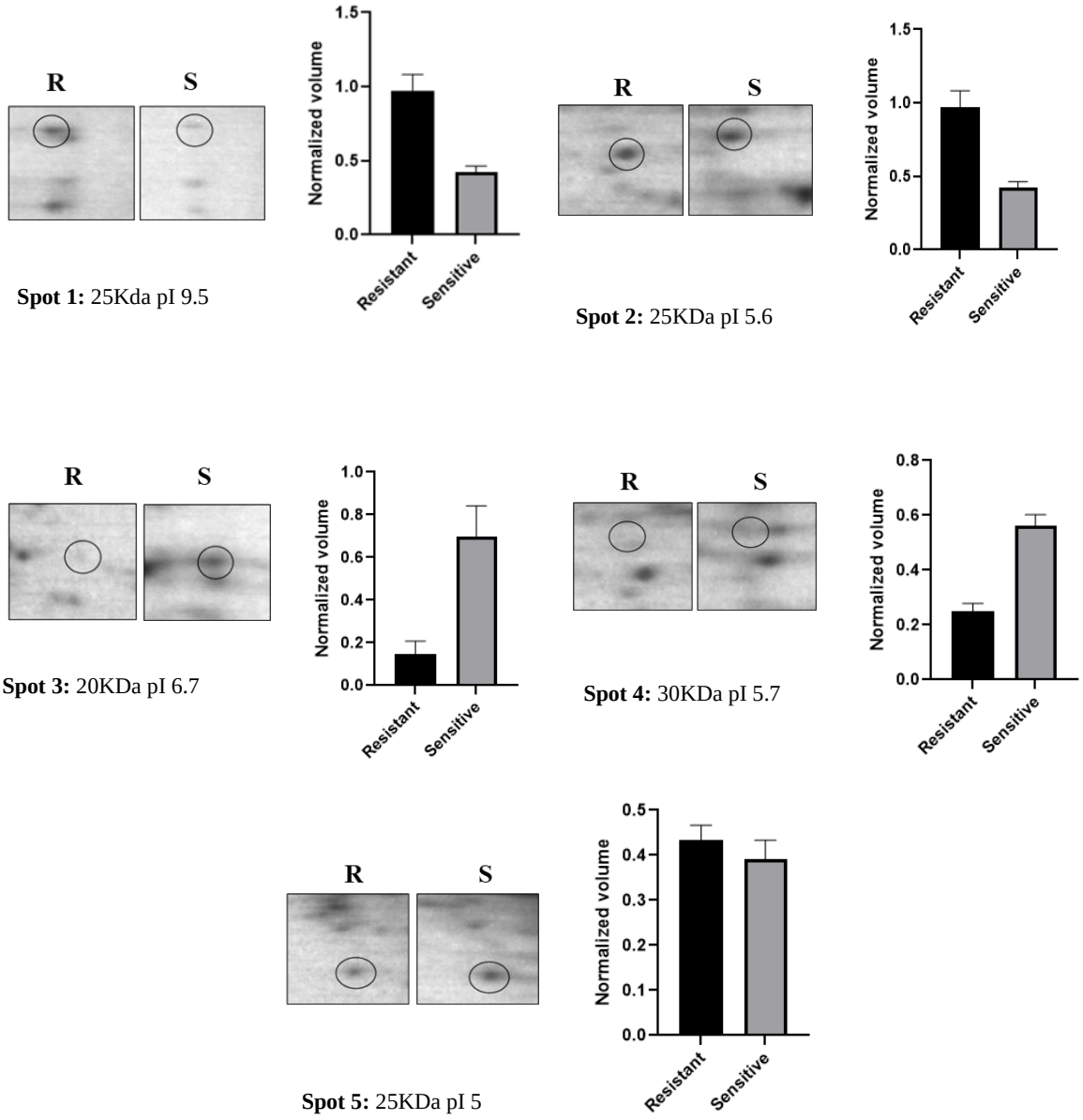
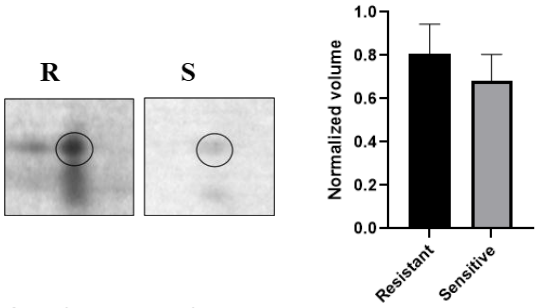
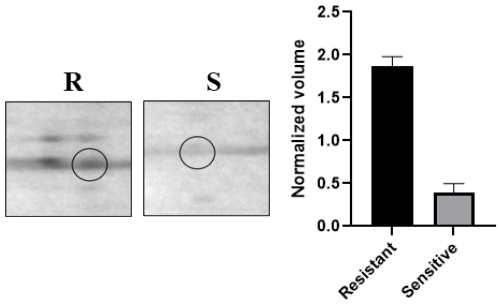


Figure 4.12: Expression graphs of differentially expressed protein of U87 Resistant and U87 Sensitive cell lines. The graph based on normalized volume of spots made with graph pad prism 9.3. The statistics of t-test paired were applied and significant p value (≤0.05) were

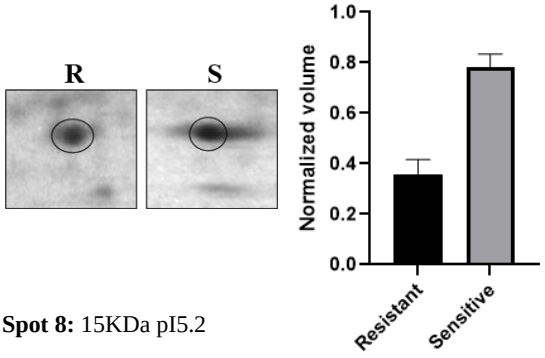
found. Spots 1,2 and 5 have increased expression in resistant U87 cell lines while 3 and 4 have decreased expression in resistant U87 cell lines.



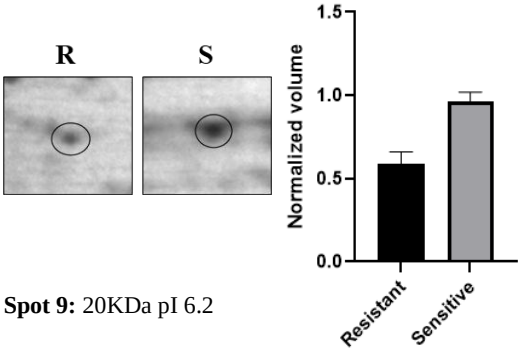
Spot 6: 15KDa pI 9.4



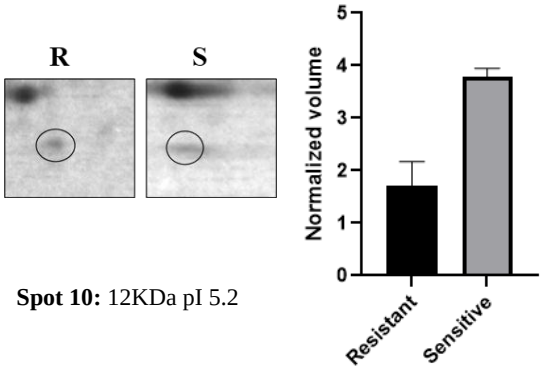
Spot 7: 37KDa pI 9.5



Spot 8: 15KDa pI5.2



Spot 9: 20KDa pI 6.2



Spot 10: 12KDa pI 5.2

Figure 4.13: Expression graphs of differentially expressed protein of U87 Resistant and U87 Sensitive cell lines. The graph based on normalized volume of spots made with graph pad prism 9.3. The statistics of t-test paired were applied and significant p value (≤0.05) were

found. Spots 1,2 and 5 have increased expression in resistant U87 cell lines while 3 and 4 have decreased expression in resistant U87 cell lines.

2.4 Prediction of Glycosylation Sites

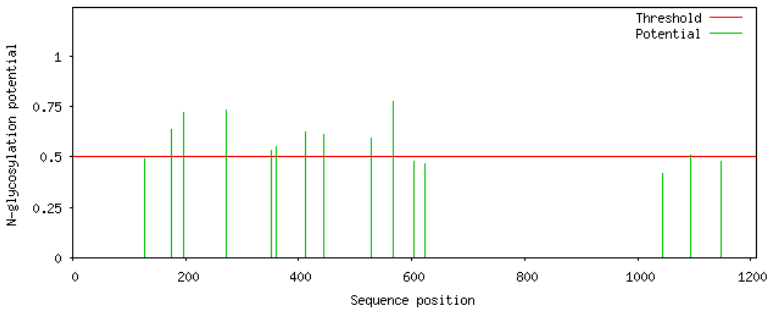
The selected target proteins of GBM; Hsp27, EGFR, TNF-α, Annexin A2, IL17A, galectin-1 were checked for post translational modification of Glycosylation with online glycosylation prediction software NetNglyc. The FASTA sequence of the proteins retrieved from uniport was added in NetNGlyc software which gave the predicted sites. The prediction was based on Asparagine that comes in the Asn-Xaa-Ser/Thr sequon. The prediction of glycosylation on asparagine that was not in the mentioned sequon was not selected because it is highly

PROTEINS	UNIPROT KB	POSITION	SCORE	N-GLYCOSYLATION
EGFR	P00533	175	0.631	++
		196	0.7183	++

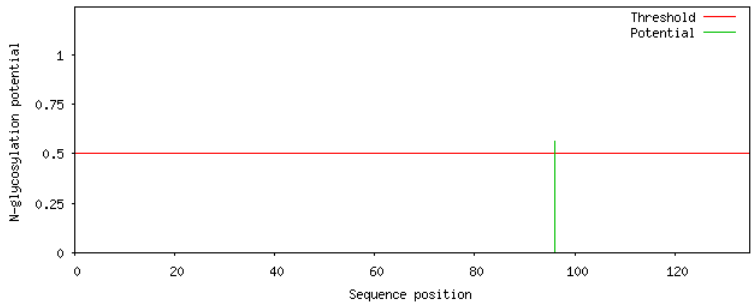
unlikely for N-glycan to attach on glycoprotein residues on other asparagines sequon. The threshold of selecting the prediction sites was above 0.5.

		352	0.5327	+
		361	0.5524	+
		413	0.6249	++
		444	0.6102	+
		528	0.5906	+
		568	0.7723	+++
		1096	0.5062	+
HSP27	P04792	No prediction		
TNF-A	P01375	No prediction		
ANNEXIN A2	P07355	62	0.7073	++
IL17A	Q16552	68	0.5336	+
GALECTIN-1	P09382	96	0.5650	+

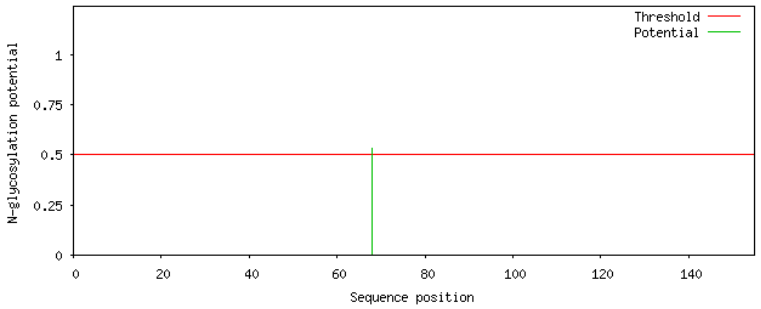
Table 4.4: Predicted positions of N-glycosylation along the length of protein. The + signs indicate the potential of glycosylation by the software, where one + indicates prediction score above 0.5. Double ++ means the threshold above 0.75 or with jury agreement 9/9 which is based on software 9 criteria.



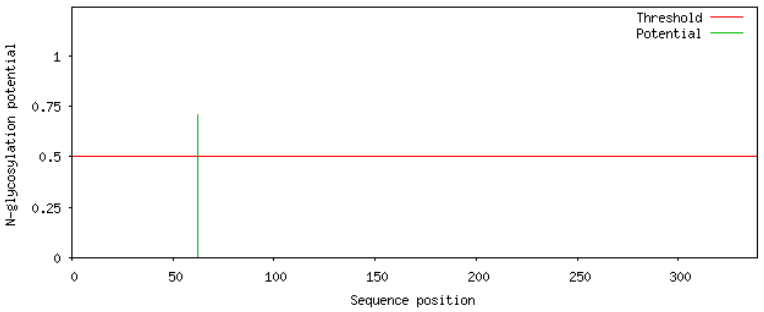
EGFR



Annexin A2



IL17A



Galectin 1

Figure 4.14 A graphic illustration of glycosylation potentials across the protein sequence length. (x-axis represents protein length from N- to C-terminal). A position with a potential (vertical lines) crossing the threshold (horizontal line at 0.5) is predicted glycosylated

2.5 Differential Protein Glycosylation in GBM

The protein lysate from U87 Sensitive and Resistant cell lines was subjected to SDS-PAGE. The gels were subjected to two different strains, Coomassie which stain the whole protein and Glyco stain which stain the glycan to study glycosylation pattern in both the sensitive and resistant variants of U87MG cell lines. The differentially expressed bands in Coomassie stained and Glyco stained were noted. The gels are represented in figure 4.15 and 4.16. Image Lab software was employed for the quantitation of gels based on densitometric analysis. The bands of 43KDa were found to be differentially expressed in Glyco stain. The graphs for the differentially expressed band are shown in figure 4.17 and 4.18. 43KDa band were also differentially expressed in Coomassie but the difference in expression in glycosylation is more prominent in this size protein. So, the 43KDa protein were found to be more glycosylated in resistant U87MG cell lines as compared to sensitive ones.

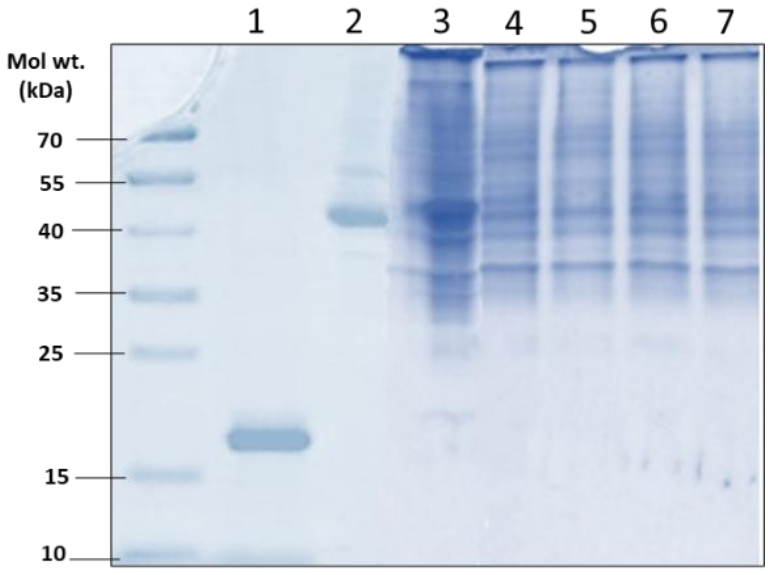


Figure 4.15: Representative gel for extracted proteins of U87-MG cell lines separated on 12.5% SDS-PAGE and stained with Coomassie brilliant blue. Lane 1: -ve control of glycol protein stain, Lane 2 +ve control of glycol protein stain, Lane 3: healthy mice brain

protein 50 ug, Lane 4 U87 sensitive protein 50ug, Lane 5 U87 sensitive protein 75 ug, Lane 6 U87 Resistant protein 50 ug, Lane 7 U87 resistant protein 75 ug.

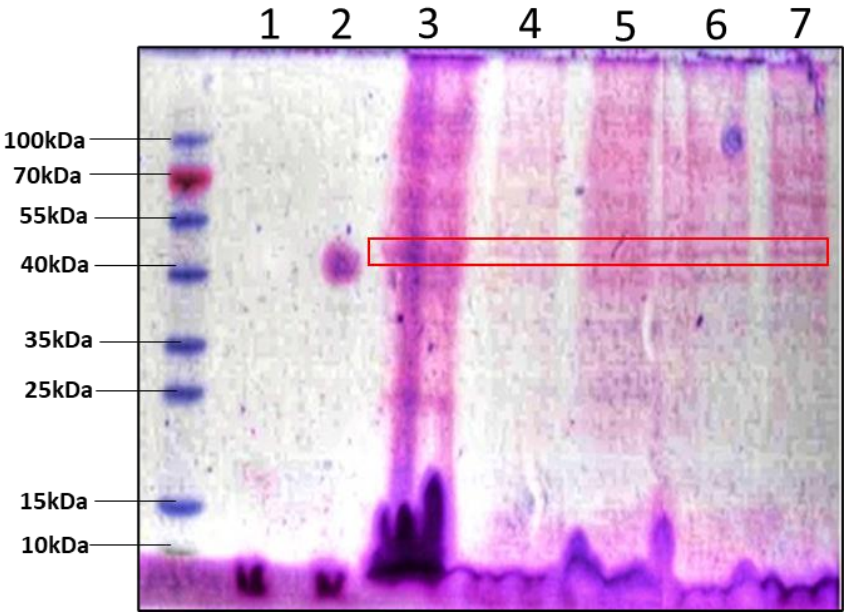


Figure 4.16: Representative gel for glycosylated proteins expressed in U87-MG cell lines. Lane 1: -ve control of glycol protein stain, Lane 2 +ve control of glycol protein stain, Lane 3: healthy mice brain protein 50 ug, Lane 4 U87 sensitive protein 50ug, Lane 5 U87 sensitive protein 75 ug, Lane 6 U87 resistant protein 50 ug, Lane 7 U87 resistant protein 75 ug.

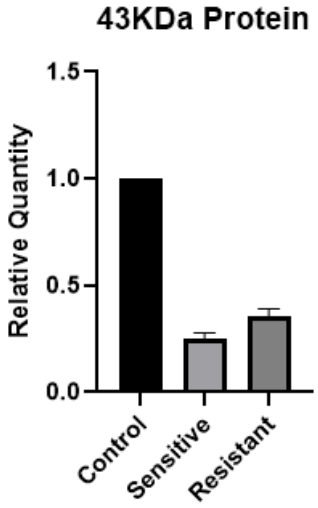


Figure 4.17: Relative quantity graph for the differentially expressed 43Kda protein in U87-MG cell lines. relative quantity obtained with Image Lab software and graph made with Prism 9.3, showing differential expression of 43KDa protein in sensitive and resistance cell lines.

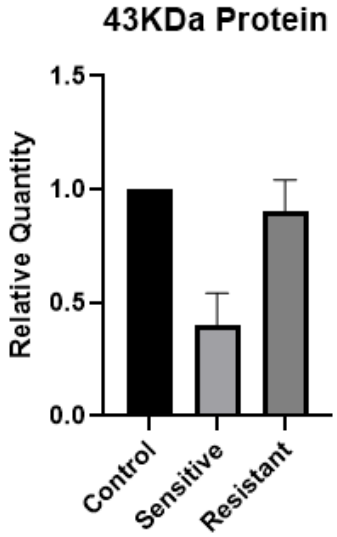


Figure 4.18: Relative quantity graph for the differentially expressed 43Kda glycosylated protein in U87-MG cell lines. Relative quantity of protein obtained with Image Lab software and graph made with prism 9.3 showing differential expression of 43KDa protein in sensitive and resistance cell lines.

Discussion

This study explores the potential of RA as a prospective drug for GBM. The study has explored various aspects of GBM pathology and both in silico and invitro studies showed significant findings. The in silico assessment was performed with aberrantly expressed proteins based on their functional aspects and associated pathway in GBM. Proteins that were investigated in this study for their interaction potential with RA includes EGFR, Hsp27, TNF-α, Annexin A2, IL17A, and galectin-1. EGFR is a transmembrane tyrosine kinase receptor, in normal cells plays role in cell signaling, division and survival. Under pathological conditions, it is associated with tumorigenesis especially in GBM, lungs and breast cancer. It is considered as biomarker of resistance in cancers (Sigismund et al., 2018). It is upregulated in 57.4% of GBM cases. Downregulation of it by RA based on the docking results could stop its abnormal signaling cascades which are otherwise activated in cancer cells and can have improving role in GBM (An et al., 2018a). Hsp27, heat shock protein, play's role in inhibition of apoptosis, cell development and differentiation. In cancer cells its upregulation is linked with proliferation, differentiation, metastasis and prevent apoptosis. The over expression of this gene is also linked with the development of resistance in GBM. RA exhibited strongest binding affinity with Hsp27 compared to rest. Studies already show considerable tumor reduction when administered with Hsp-27 inhibitor. RA can form stable conformations with Hsp-27, which implies it can inhibit its role and hence reduce GBM volume and metastasis (Vidyasagar et al., 2012). TNF-α, tumor necrosis factor is transmembrane protein receptor plays role in inflammatory pathways, immune system and cell signaling. RA also showed stable interactions with it. Inhibition of TNF-a along with EGFR inhibitors showed superior results in GBM especially in resistant GBM model compared to TMZ. Since our compound of interest RA has binding potential with both of the target proteins it can be speculated to be more efficacious then standard drug TMZ rather

than combination of two inhibitors (Guo et al., 2019). Annexin A2 inhibits NF-kβ signal transduction pathway and its upregulation is exploited by cancerous cells to avoid apoptosis and proliferate. Level of annexin in directly related to degree of malignancy. The gene coding annexin a2 knock out results in decreases glioma migration. Hence, its down regulation is the aim. RA showed stable conformation and 3 hydrogen bonds with annexin, so it can be deduced that RA will also result in decreased migration based on its interaction with annexin a2 (Zhai et al., 2011). IL17A induce vascularity and angiogenesis at the inflammation site which aggravates the GBM, it does this through PI3K/AKT signaling pathway and its up-regulation is a prominent biomarker of GBM (Zheng et al., 2019). Docking with RA suggest it can form stable conformation and might down regulate IL17A, its inhibitor has reported increased survival, decreased tumor growth and tumor hypoxia on U87-MG (M. S. S. Khan et al., 2017) and same results could be expected from RA. Galectin 1 has its role in functions like cell proliferation, cell adhesion, tumor metastasis, immunoregulatory effects and apoptosis (Puchades et al., 2007). The docking results gave promising binding affinity scores and covalent H-bond interactions with these target proteins which implies that RA has potential to interact with these compounds and might be able normalize their expression. All these drug targets normalized together with one compound makes RA a promising drug candidate.

Docking study with these proteins was also performed on standard drug Temozolomide. The known mechanism of action of this drug is DNA-Alkylation (Lee, 2016). However, from the results it also showed interactions and good binding affinity scores with selected target proteins which suggests TMZ has mode of action through these biomarkers of GBM. The comparison of TMZ and RA showed RA has better binding affinities with the GBM target proteins.

U87-MG cell lines are common GBM experimental model (Li et al., 2017). The standard U87 cell lines are sensitive to TMZ drug. A resistant variant of U87 cell lines was developed by inducing increased concentration of TMZ 980uM for 14-21 days. TMZ mode of action is through DNA alkylation O6-G, which triggers mismatch repair mechanism in cell and on recognizing a recurring error induce apoptosis. Increased O^6-methylguanine methyltransferase (MGMT), repairs the methylated DNA and hence act as antagonist of TMZ. There are other unknown mechanism causing development of resistance in GBM, independent of their MGMT levels (Sun et al., 2012). Comparative analysis of proteins of the sensitive and resistant variant are performed in this study in an attempt to understand the mechanism undying the development of resistance which is crucial for the development of better treatment strategies in future. Most studies conducted are at genomic and transcriptional level. This study takes a proteomic approach, which can uncover such mechanisms by directly addressing the functional effectors of cellular, disease and treatment processes.

This investigation was able to identify 10 significantly ($p>0.05$) differentially expressed proteins between sensitive and resistant U87 cell lines. Out of these 5 proteins were up regulated and 5 were down regulated in U87 resistant compared to U87 sensitive lines. Spot 1,2,5,6 and 7 were up regulated in resistant U87 cell lines (Figure 13, 14).

A spot of approx. 43 KDa found to be up regulated protein in resistant U87-MG compared to sensitive which on student's t-test gave the highly significant value ($P=0.0004$). IL17A which was the selected protein target of interest for GBM is also a 40 KDa protein and is significantly up regulated in GBM. The upregulation of IL17A substantially effects PI3K/AKT signaling pathway (Zheng et al., 2019). Identification of this 40 kDa protein through mass spectrometry will provide the further affirmation of this identified protein. The differential expression and the identification of the identified spots will allow us to determine

which pathways are determinant to neuronal dysfunction and to establish a timeline for disease progression.

Moreover, altered Glycosylation is a hall mark aberration observed in various cancers. The in-silico prediction on plausible glycosylated sites in selected target proteins, observed in this study provides clues to the involvement of altered glycosylation in expression alterations in the observed differentially expressed proteins. The target proteins studied through in silico analysis showed several predicted sites. EGFR that exhibites 9 predicted sites, on deglycosylation shows the decrease of 40 KDa then the normal glycosylated protein. EGFR through domain of tyrosine kinase regulates cell behavior. Deglycosylated EGFR is unable to start cell proliferation and are not fully phosphorylated even with intact tyrosine kinase, highlights the importance of glycosylation (Wang et al., 2001). The glycosylation pattern was also investigated in both variants of U87 cell lines where the differential glycosylation pattern of a 43 KDa were observed to be highly glycosylated in resistant U87 lines as compared to sensitive U87 line. IL17A has molecular weight of 35Kda, single N-glycosylation site at Asn68 and its variable glycosylation has been cautiously attributed to altered inflammatory response (Geoghegan et al., 2013) Similarly the glycosylated bands found in this study would be of proteins around 40 KDa and this could possibly include IL17A. This altered glycosylation would alter the protein structure and hence all the following cascade of protein activity and other proteins of same molecular weight. The mass spectrometry of these band could identify proteins with dysregulated glycosylation which could offer us insight on GBM resistance development.

The present research work provides the basis for further elucidation of these preliminary findings on differentially expressed proteins of U87-MG cell lines.

Conclusion and Future Prospects

The study conducted affirms the hypothesis that Rosmarinic Acid could be a prospective drug and should be validated further in vitro and in vivo experiments.

Differential expression of proteins was observed and a total of 10 significantly different expressed proteins were identified between U87-MG sensitive and resistant. Mass spectrometry should be performed of the identified spots to find the particular proteins in future. This would help understand the molecular mechanism behind the development of resistance against standard drug. They can serve as biomarkers and drug targets.

Overall, we posit that future studies need to focus on differentially expressed proteins that may aid in to unravel plausible molecular mechanisms involved in the development of drug resistance to TMZ which ultimately leads to strategizing effective treatment options for GBM.

References

Abdullah, Umm E. Hani, Altaf Ali Laghari, Muhammad Usman Khalid, Huzaifa Bin Rashid, Adnan Abdul Jabbar, Fatima Mubarak, Asim Hafiz, Shahzad Shamim, and Syed Ather Enam. 2019. "Current Management of Glioma in Pakistan." *Glioma* 2(3):139. doi: 10.4103/glioma.glioma_15_19.

An, Zhenyi, Ozlem Aksoy, Tina Zheng, Qi-Wen Fan, and William A. Weiss. 2018. "Epidermal Growth Factor Receptor (EGFR) and EGFRvIII in Glioblastoma (GBM): Signaling Pathways and Targeted Therapies." *Oncogene* 37(12):1561–75. doi: 10.1038/s41388-017-0045-7.

Anjum, Komal, Bibi Ibtesam Shagufta, Syed Qamar Abbas, Seema Patel, Ishrat Khan, Sayed Asmat Ali Shah, Najeeb Akhter, and Syed Shams ul Hassan. 2017. "Current Status and Future Therapeutic Perspectives of Glioblastoma Multiforme (GBM) Therapy: A Review." *Biomedicine & Pharmacotherapy* 92:681–89. doi: 10.1016/j.biopha.2017.05.125.

Balça-Silva, Joana, Diana Matias, Anália Do Carmo, Luiz Gustavo Dubois, Ana Cristina Gonçalves, Henrique Girão, Nathalie Henriques Silva Canedo, Ana Helena Correia, Jorge Marcondes De Souza, Ana Bela Sarmento-Ribeiro, Maria Celeste Lopes, and Vivaldo Moura-Neto. 2017. "Glioblastoma Entities Express Subtle Differences in Molecular Composition and Response to Treatment." *Oncology Reports* 38(3):1341–52. doi: 10.3892/or.2017.5799.

Brada, Michael, Sally Stenning, Rhian Gabe, Lindsay Thompson, David Levy, Roy Rampling, Sara Erridge, Frank Saran, Rao Gattamaneni, Kirsten Hopkins, Sarah Beall, Vincent Collins, and Siow-Ming Lee. 2010. "Temozolomide Versus Procarbazine, Lomustine, and Vincristine in Recurrent High-Grade Glioma." *Journal of Clinical Oncology: Official Journal of the American Society of Clinical Oncology* 28:4601–8. doi: 10.1200/JCO.2009.27.1932.

Bradford, Marion M. 1976. "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding." *Analytical Biochemistry* 72(1):248–54. doi: 10.1016/0003-2697(76)90527-3.

Cabrera, Alvin R., John P. Kirkpatrick, John B. Fiveash, Helen A. Shih, Eugene J. Koay, Stephen Lutz, Joshua Petit, Samuel T. Chao, Paul D. Brown, Michael Vogelbaum, David A.

Reardon, Arnab Chakravarti, Patrick Y. Wen, and Eric Chang. 2016. "Radiation Therapy for Glioblastoma: Executive Summary of an American Society for Radiation Oncology Evidence-Based Clinical Practice Guideline." *Practical Radiation Oncology* 6(4):217–25. doi: 10.1016/j.prro.2016.03.007.

Camby, Isabelle, Marie Le Mercier, Florence Lefranc, and Robert Kiss. 2006. "Galectin-1: A Small Protein with Major Functions." *Glycobiology* 16(11):137R-157R. doi: 10.1093/glycob/cwl025.

Cattaneo, Lucia, Rosella Cicconi, Giuseppina Mignogna, Alessandra Giorgi, Maurizio Mattei, Giulia Graziani, Rosalia Ferracane, Alessandro Grosso, Patrizia Aducci, M. Eugenia Schininà, and Mauro Marra. 2015. "Anti-Proliferative Effect of Rosmarinus Officinalis L. Extract on Human Melanoma A375 Cells." *PLOS ONE* 10(7):e0132439. doi: 10.1371/journal.pone.0132439.

Chien, Ching-Hsuan, Chi-Chang Chang, Shih-Huan Lin, Chi-Wei Chen, Zong-Han Chang, and Yen-Wei Chu. 2020. "N-GlycoGo: Predicting Protein N-Glycosylation Sites on Imbalanced Data Sets by Using Heterogeneous and Comprehensive Strategy." *IEEE Access* 8:165944–50. doi: 10.1109/ACCESS.2020.3022629.

Chumbalkar, Vaibhav C., Chanumolu Subhashini, Vishnu M. Dhople, Curam S. Sundaram, Medicharla V. Jagannadham, Kafnam N. Kumar, Pasumarti N. B. S. Srinivas, Ravinutala Mythili, Mulla K. Rao, Mahesh J. Kulkarni, Sridevi Hegde, Alangar S. Hegde, Cini Samual, Vani Santosh, Lalji Singh, and Ravi Sirdeshmukh. 2005. "Differential Protein Expression in Human Gliomas and Molecular Insights." *PROTEOMICS* 5(4):1167–77. doi: 10.1002/pmic.200401202.

Cutler, Paul, ed. 2004. *Protein Purification Protocols*. 2nd ed. Humana Press.

Geoghegan, Kieran F., Xi Song, Lise R. Hoth, Xidong Feng, Suman Shanker, Amira Quazi, Deborah P. Luxenberg, Jill F. Wright, and Matthew C. Griffor. 2013. "Unexpected Mucin-Type O-Glycosylation and Host-Specific N-Glycosylation of Human Recombinant Interleukin-17A Expressed in a Human Kidney Cell Line." *Protein Expression and Purification* 87(1):27–34. doi: 10.1016/j.pep.2012.09.013.

Ghasemzadeh Rahbardar, Mahboobeh, and Hossein Hosseinzadeh. 2020. "Therapeutic Effects of Rosemary (Rosmarinus Officinalis L.) and Its Active Constituents on Nervous

System Disorders." *Iranian Journal of Basic Medical Sciences* 23(9):1100–1112. doi: 10.22038/ijbms.2020.45269.10541.

Grech, Neil, Theresia Dalli, Sean Mizzi, Lara Meilak, Neville Calleja, and Antoine Zrinzo. 2020. "Rising Incidence of Glioblastoma Multiforme in a Well-Defined Population." *Cureus* 12(5). doi: 10.7759/cureus.8195.

Guo, Gao, Ke Gong, Vineshkumar Thidil Puliyappadamba, Nishah Panchani, Edward Pan, Bipasha Mukherjee, Ziba Damanwalla, Sabrina Bharia, Kimmo J. Hatanpaa, David E. Gerber, Bruce E. Mickey, Toral R. Patel, Jann N. Sarkaria, Dawen Zhao, Sandeep Burma, and Amyn A. Habib. 2019. "Efficacy of EGFR plus TNF Inhibition in a Preclinical Model of Temozolomide-Resistant Glioblastoma." *Neuro-Oncology* 21(12):1529–39. doi: 10.1093/neuonc/noz127.

Gupta, Manoj Kumar, Ravindra Varma Polisetty, Rakesh Sharma, Raksha A. Ganesh, Harsha Gowda, Aniruddh K. Purohit, Praveen Ankathi, Komal Prasad, Kiran Mariswamappa, Akhila Lakshmikantha, Megha S. Uppin, Challa Sundaram, Poonam Gautam, and Ravi Sirdeshmukh. 2019. "Altered Transcriptional Regulatory Proteins in Glioblastoma and YBX1 as a Potential Regulator of Tumor Invasion." *Scientific Reports* 9(1):10986. doi: 10.1038/s41598-019-47360-9.

Gupta, Mukta, Naresh Singh, Monica Gulati, Reena Gupta, Kalvatala Sudhakar, and Bhupinder Kapoor. 2021. "Herbal Bioactives in Treatment of Inflammation: An Overview." *South African Journal of Botany* 143:205–25. doi: 10.1016/j.sajb.2021.07.027.

Hanif, Farina, Kanza Muzaffar, Kahkashan Perveen, Saima M. Malhi, and Shabana U. Simjee. 2017. "Glioblastoma Multiforme: A Review of Its Epidemiology and Pathogenesis through Clinical Presentation and Treatment." *Asian Pacific Journal of Cancer Prevention APJCP* 18(1):3–9. doi: 10.22034/APJCP.2017.18.1.3.

Holland, Eric C. 2000. "Glioblastoma Multiforme: The Terminator." *Proceedings of the National Academy of Sciences* 97(12):6242–44. doi: 10.1073/pnas.97.12.6242.

Hong, Xin, Khalil Chedid, and Steven N. Kalkanis. 2012. "Glioblastoma Cell Line-Derived Spheres in Serum‐containing Medium versus Serum-Free Medium: A Comparison of Cancer Stem Cell Properties." *International Journal of Oncology* 41(5):1693–1700. doi: 10.3892/ijo.2012.1592.

Hottinger, Andreas F., Roger Stupp, and Krisztian Homicsko. 2014. "Standards of Care and Novel Approaches in the Management of Glioblastoma Multiforme." *Chinese Journal of Cancer* 33(1):32–39. doi: 10.5732/cjc.013.10207.

Ji, Hong. 2010. "Lysis of Cultured Cells for Immunoprecipitation." *Cold Spring Harbor Protocols* 2010(8). doi: 10.1101/pdb.prot5466.

Kanderi, Tejaswi, and Vikas Gupta. 2021. "Glioblastoma Multiforme." *StatPearls*. Treasure Island (FL): StatPearls Publishing. //www.ncbi.nlm.nih.gov/books/NBK558954/.

Khalid, Anibah, Umme Aimen Abbasi, Sanila Amber, null Sumera, Fatima Javed Mirza, Muhammad Asif, Aneela Javed, and Saadia Zahid. 2020. "Methylphenidate and Rosmarinus Officinalis Improves Cognition and Regulates Inflammation and Synaptic Gene Expression in AlCl3-Induced Neurotoxicity Mouse Model." *Molecular Biology Reports* 47(10):7861–70. doi: 10.1007/s11033-020-05864-y.

Khan, Md Shamsuddin Sultan, Mohammad Iqbal, Muhammad Asif, Tabinda Azam, Majed Al-Mansoub, Rosenani Haque, Mohamed B. Khadeer Ahamed, Aman Shah Abdul Majid, and Amin Malik Shah Abdul Majid. 2019. "Anti-GBM Potential of Rosmarinic Acid and Its Synthetic Derivatives via Targeting IL17A Mediated Angiogenesis Pathway." *Journal of Angiotherapy* 3:097–122. doi: 10.25163/angiotherapy.21206512012110519.

Khan, Mohsin Ahmad, Nadeem Ahmed, Muhammad Islam Khan, Ahmad Usman Zafar, Saad Tahir, Muhammad Sohail Anjum, Muhammad Ali, Faidad Khan, and Tayyab Husnain. 2017. "Bioactivity Studies of Huh-7 Cells Derived Human Epidermal Growth Factor Expressed in Pichia Pastoris." *Bioscience, Biotechnology, and Biochemistry* 81(6):1114–19. doi: 10.1080/09168451.2017.1295802.

Kumar, Swatantra, Vimal K. Maurya, Anil K. Prasad, Madan L. B. Bhatt, and Shailendra K. Saxena. 2020. "Structural, Glycosylation and Antigenic Variation between 2019 Novel Coronavirus (2019-NCoV) and SARS Coronavirus (SARS-CoV)." *Virus Disease* 31(1):13–21. doi: 10.1007/s13337-020-00571-5.

Lee, Eunjee, Raymund L. Yong, Patrick Paddison, and Jun Zhu. 2018. "Comparison of Glioblastoma (GBM) Molecular Classification Methods." *Seminars in Cancer Biology* 53:201–11. doi: 10.1016/j.semcancer.2018.07.006.

Lee, Sang Y. 2016. "Temozolomide Resistance in Glioblastoma Multiforme." *Genes & Diseases* 3(3):198–210. doi: 10.1016/j.gendis.2016.04.007.

Lou, Weiyang, Bisha Ding, Liang Xu, and Weimin Fan. 2019. "Construction of Potential Glioblastoma Multiforme-Related MiRNA-MRNA Regulatory Network." *Frontiers in Molecular Neuroscience* 12. doi: 10.3389/fnmol.2019.00066.

Mendez-Huergo, Santiago P., Pablo F. Hockl, Juan C. Stupirski, Sebastián M. Maller, Luciano G. Morosi, Nicolás A. Pinto, Ana M. Berón, Jorge L. Musuruana, Gustavo G. Nasswetter, Javier A. Cavallasca, and Gabriel A. Rabinovich. 2019. "Clinical Relevance of Galectin-1 and Galectin-3 in Rheumatoid Arthritis Patients: Differential Regulation and Correlation With Disease Activity." *Frontiers in Immunology* 9. doi: 10.3389/fimmu.2018.03057.

Nusinow, David P., John Szpyt, Mahmoud Ghandi, Christopher M. Rose, E. Robert McDonald, Marian Kalocsay, Judit Jané-Valbuena, Ellen Gelfand, Devin K. Schweppe, Mark Jedrychowski, Javad Golji, Dale A. Porter, Tomas Rejtar, Y. Karen Wang, Gregory V. Kryukov, Frank Stegmeier, Brian K. Erickson, Levi A. Garraway, William R. Sellers, and Steven P. Gygi. 2020. "Quantitative Proteomics of the Cancer Cell Line Encyclopedia." *Cell* 180(2):387-402.e16. doi: 10.1016/j.cell.2019.12.023.

Ozdemir, Meryem Damla, and Dilek Gokturk. 2018. "The Effect of Rosmarinus Officinalis and Chemotherapeutic Etoposide on Glioblastoma (U87 MG) Cell Culture." *Turkish Neurosurgery* 28(6):853–57. doi: 10.5137/1019-5149.JTN.20401-17.3.

Pei, Jian, Kyung-Sub Moon, SangO Pan, Kyung-Hwa Lee, Hyang-Hwa Ryu, Tae-Young Jung, In-Young Kim, Woo-Yeol Jang, Chae-Hun Jung, and Shin Jung. 2014. "Proteomic Analysis between U87MG and U343MG-A Cell Lines: Searching for Candidate Proteins for Glioma Invasion." *Brain Tumor Research and Treatment* 2(1):22–28. doi: 10.14791/btrt.2014.2.1.22.

Pitti, Thejkiran, Ching-Tai Chen, Hsin-Nan Lin, Wai-Kok Choong, Wen-Lian Hsu, and Ting-Yi Sung. 2019. "N-GlyDE: A Two-Stage N-Linked Glycosylation Site Prediction Incorporating Gapped Dipeptides and Pattern-Based Encoding." *Scientific Reports* 9(1):15975. doi: 10.1038/s41598-019-52341-z.

Puchades, Maja, Carol L. Nilsson, Mark R. Emmett, Kenneth D. Aldape, Yongjie Ji, Frederick F. Lang, Ta-Jen Liu, and Charles A. Conrad. 2007. "Proteomic Investigation of

Glioblastoma Cell Lines Treated with Wild-Type P53 and Cytotoxic Chemotherapy Demonstrates an Association between Galectin-1 and P53 Expression." *Journal of Proteome Research* 6(2):869–75. doi: 10.1021/pr060302l.

Pugalenthi, Ganesan, Varadharaju Nithya, Kuo-Chen Chou, and Govindaraju Archunan. 2020. "Nglyc: A Random Forest Method for Prediction of N-Glycosylation Sites in Eukaryotic Protein Sequence." *Protein and Peptide Letters* 27(3):178–86. doi: 10.2174/0929866526666191002111404.

Qi, Songtao, and Yawei Liu. 2016. "Differences in Protein Expression between the U251 and U87 Cell Lines." *Turkish Neurosurgery*. doi: 10.5137/1019-5149.JTN.17746-16.1.

Ramanauskiene, Kristina, Raimondas Raudonis, and Daiva Majiene. 2016. "Rosmarinic Acid and Melissa Officinalis Extracts Differently Affect Glioblastoma Cells." *Oxidative Medicine and Cellular Longevity* 2016 1564257. doi: 10.1155/2016/1564257.

Reddy, Panga, Aishwarya Rao, Darpan Malhotra, Samridhi Sharma, Ravinder Kumar, Rekha Jain, Kishore Gollapalli, Namita Pendharkar, Srikanth Rapole, and Sanjeeva Srivastava. 2014. "A Simple Protein Extraction Method for Proteomic Analysis of Diverse Biological Specimens." *Current Proteomics* 10(4):298–311. doi: 10.2174/15701646113106660004.

Rizvi, Syed Mohd. Danish, Shazi Shakil, and Mohd. Haneef. 2013. "A Simple Click by Click Protocol to Perform Docking: AutoDock 4.2 Made Easy for Non-Bioinformaticians." *EXCLI Journal* 12:831–57.

Roth, Ziv, Galit Yehezkel, and Isam Khalaila. 2012. "Identification and Quantification of Protein Glycosylation." *International Journal of Carbohydrate Chemistry* 2012: e640923. doi: 10.1155/2012/640923.

Saraswathy, Nachimuthu, and Ponnusamy Ramalingam. 2011. "10 - Introduction to Proteomics." *Concepts and Techniques in Genomics and Proteomics*, *Woodhead Publishing Series in Biomedicine*,147–58 edited by N. Saraswathy and P. Ramalingam. Woodhead Publishing.

Sayed, M. Mumtaz, Bhardwaj Gautam, Goswami Shikha, Kumar Tonk Rajiv, K. Goyal Ramesh, Abu-Izneid Tareq, and Hyder Pottoo Faheem. 2021. "Management of Glioblastoma Multiforme by Phytochemicals: Applications of Nanoparticle-Based Targeted Drug Delivery System." *Current Drug Targets* 22(4):429–42. doi:10.1016/j.yexmp.2008.12.004

Seeliger, Daniel, and Bert L. de Groot. 2010. "Ligand Docking and Binding Site Analysis with PyMOL and Autodock/Vina." *Journal of Computer-Aided Molecular Design* 24(5):417–22. doi: 10.1007/s10822-010-9352-6.

Şengelen, Aslıhan, and Evren Önay-Uçar. 2018. "Rosmarinic Acid and SiRNA Combined Therapy Represses Hsp27 (HSPB1) Expression and Induces Apoptosis in Human Glioma Cells." *Cell Stress and Chaperones* 23(5):885–96. doi: 10.1007/s12192-018-0896-z.

Shergalis, Andrea, Armand Bankhead, Urarika Luesakul, Nongnuj Muangsin, and Nouri Neamati. 2018. "Current Challenges and Opportunities in Treating Glioblastoma." *Pharmacological Reviews* 70(3):412–45. doi: 10.1124/pr.117.014944.

Sigismund, Sara, Daniele Avanzato, and Letizia Lanzetti. 2018. "Emerging Functions of the EGFR in Cancer." *Molecular Oncology* 12(1):3–20. doi: 10.1002/1878-0261.12155.

Smoll, Nicolas R., Karl Schaller, and Oliver P. Gautschi. 2013. "Long-Term Survival of Patients with Glioblastoma Multiforme (GBM)." *Journal of Clinical Neuroscience* 20(5):670–75. doi: 10.1016/j.jocn.2012.05.040.

Stetson, Lindsay C., Jean-Eudes Dazard, and Jill S. Barnholtz-Sloan. 2016. "Protein Markers Predict Survival in Glioma Patients." *Molecular & Cellular Proteomics* 15(7):2356–65. doi: 10.1074/mcp.M116.060657.

Suk, Kyoungho. 2012. "Proteomic Analysis of Glioma Chemoresistance." *Current Neuropharmacology* 10(1):72–79. doi: 10.2174/157015912799362733.

Sun, Stella, T. S. Wong, X. Q. Zhang, Jenny K. S. Pu, Nikki P. Lee, Philip J. R. Day, Gloria K. B. Ng, W. M. Lui, and Gilberto K. K. Leung. 2012. "Protein Alterations Associated with Temozolomide Resistance in Subclones of Human Glioblastoma Cell Lines." *Journal of Neuro-Oncology* 107(1):89–100. doi: 10.1007/s11060-011-0729-8.

Taherzadeh, Ghazaleh, Abdollah Dehzangi, Maryam Golchin, Yaoqi Zhou, and Matthew P. Campbell. 2019. "SPRINT-Gly: Predicting N- and O-Linked Glycosylation Sites of Human and Mouse Proteins by Using Sequence and Predicted Structural Properties." *Bioinformatics* 35(20):4140–46. doi: 10.1093/bioinformatics/btz215.

Taylor, Olivia G., Joshua S. Brzozowski, and Kathryn A. Skelding. 2019. "Glioblastoma Multiforme: An Overview of Emerging Therapeutic Targets." *Frontiers in Oncology* 9. doi: 10.3389/fonc.2019.00963.

Tondepu, Chaitanya, and Lohitash Karumbaiah. n.d. “Glycomaterials to Investigate the Functional Role of Aberrant Glycosylation in Glioblastoma.” *Advanced Healthcare Materials* n/a(n/a):2101956. doi: 10.1002/adhm.202101956.

Trott, Oleg, and Arthur J. Olson. 2010. “AutoDock Vina: Improving the Speed and Accuracy of Docking with a New Scoring Function, Efficient Optimization and Multithreading.” *Journal of Computational Chemistry* 31(2):455–61. doi: 10.1002/jcc.21334.

Tumur, Zohra, Carlos Guerra, Peter Yanni, Ahmad Eltejaye, Christi Waer, Tursun Alkam, and Bradley S. Henson. 2015. “Rosmarinic Acid Inhibits Cell Growth and Migration in Head and Neck Squamous Cell Carcinoma Cell Lines by Attenuating Epidermal Growth Factor Receptor Signaling.” *Journal of Cancer Science & Therapy* 7(12). doi: 10.4172/1948-5956.1000376.

Veillon, Lucas, Christina Fakih, Hadi Abou-El-Hassan, Firas Kobeissy, and Yehia Mechref. 2018. “Glycosylation Changes in Brain Cancer.” *ACS Chemical Neuroscience* 9(1):51–72. doi: 10.1021/acschemneuro.7b00271.

Vidyasagar, Aparna, Nancy A. Wilson, and Arjang Djamali. 2012. “Heat Shock Protein 27 (HSP27): Biomarker of Disease and Therapeutic Target.” *Fibrogenesis & Tissue Repair* 5:7. doi: 10.1186/1755-1536-5-7.

Volkamer, Andrea, Daniel Kuhn, Thomas Grombacher, Friedrich Rippmann, and Matthias Rarey. 2012a. “Combining Global and Local Measures for Structure-Based Druggability Predictions.” *Journal of Chemical Information and Modeling* 52(2):360–72. doi: 10.1021/ci200454v.

Volkamer, Andrea, Daniel Kuhn, Thomas Grombacher, Friedrich Rippmann, and Matthias Rarey. 2012b. “Combining Global and Local Measures for Structure-Based Druggability Predictions.” *Journal of Chemical Information and Modeling* 52(2):360–72. doi: 10.1021/ci200454v.

Vredenburgh, James J., Annick Desjardins, James E. Herndon, Jeannette M. Dowell, David A. Reardon, Jennifer A. Quinn, Jeremy N. Rich, Sith Sathornsumetee, Sridharan Gururangan, Melissa Wagner, Darell D. Bigner, Allan H. Friedman, and Henry S. Friedman. 2007. “Phase II Trial of Bevacizumab and Irinotecan in Recurrent Malignant Glioma.” *Clinical Cancer Research* 13(4):1253–59. doi: 10.1158/1078-0432.CCR-06-2309.

Wang, X. Q., P. Sun, M. O'Gorman, T. Tai, and A. S. Paller. 2001. "Epidermal Growth Factor Receptor Glycosylation Is Required for Ganglioside GM3 Binding and GM3-Mediated Suppresion of Activation." *Glycobiology* 11(7):515–22. doi: 10.1093/glycob/11.7.515.

Wood, Matthew D., Gerald F. Reis, David E. Reuss, and Joanna J. Phillips. 2016. "Protein Analysis of Glioblastoma Primary and Posttreatment Pairs Suggests a Mesenchymal Shift at Recurrence." *Journal of Neuropathology and Experimental Neurology* 75(10):925–35. doi: 10.1093/jnen/nlw068.

Wu, Qinghua, Zhongxu Cao, Weiwei Xiao, Li Zhu, Qian Xie, Ling Li, Bao Zhang, and Wei Zhao. 2018a. "Study on Therapeutic Action and Mechanism of TMZ Combined with RITA Against Glioblastoma." *Cellular Physiology and Biochemistry* 51(6):2536–46. doi: 10.1159/000495923.

Wu, Qinghua, Zhongxu Cao, Weiwei Xiao, Li Zhu, Qian Xie, Ling Li, Bao Zhang, and Wei Zhao. 2018b. "Study on Therapeutic Action and Mechanism of TMZ Combined with RITA Against Glioblastoma." *Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology* 51(6):2536–46. doi: 10.1159/000495923.

Wu, Wei, Jessica L. Klockow, Michael Zhang, Famyrah Lafortune, Edwin Chang, Linchun Jin, Yang Wu, and Heike E. Daldrup-Link. 2021. "Glioblastoma Multiforme (GBM): An Overview of Current Therapies and Mechanisms of Resistance." *Pharmacological Research* 171:105780. doi: 10.1016/j.phrs.2021.105780.

Yi, Guo-zhong, Wei Xiang, Wen-yan Feng, Zi-yang Chen, Yao-min Li, Sheng-ze Deng, Man-lan Guo, Liang Zhao, Xue-gang Sun, Min-yi He, Song-tao Qi, and Ya-wei Liu. 2018. "Identification of Key Candidate Proteins and Pathways Associated with Temozolomide Resistance in Glioblastoma Based on Subcellular Proteomics and Bioinformatical Analysis." *BioMed Research International* 2018:1–12. doi: 10.1155/2018/5238760.

Zhai, Haiyan, Suchitra Acharya, Iordanis Gravanis, Saira Mehmood, Roberta J. Seidman, Kenneth R. Shroyer, Katherine A. Hajjar, and Stella E. Tsirka. 2011. "Annexin A2 Promotes Glioma Cell Invasion and Tumor Progression." *The Journal of Neuroscience* 31(40):14346–60. doi: 10.1523/JNEUROSCI.3299-11.2011

Appendix

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MOLECULAR DOCKING ANALYSIS OF ROSMARINIC ACID WITH GBM DRUG TARGETS

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Abstract:

Glioblastoma multiforme (GBM) is a tumor of glial origin and is most malignant, aggressive, and prevalent with highest mortality rate in adult brain cancer. The clinical treatment available is surgical resection of tumor followed with standard drug Temozolomide (TMZ). The current standard treatment has major limitations with 50% of patients' resistance to TMZ and average survival of 15 months after diagnosis. This underscores the need of investigating novel potential drugs and their targets in GBM. Rosmarinic acid (RA), natural constituent of Lamiaceae plants have reported neuropharmacological and anti-cancerous properties. This study seeks to investigate the binding and interacting potential of rosmarinic acid with aberrant expressing proteins in GBM. This study evaluates the molecular interaction of rosmarinic acid to following target protein: Hsp27, EGFR, TNF-α, Annexin A2, IL17A, galectin-1 as protein-ligand interactions play a vital role in drug design. Automated docking studies were performed utilizing PyRx tool powered by auto dock vina to provide useful insights into rosmarinic acid bindings to GBM potential targets. The results show encouraging therapeutic potential of RA against targeted proteins with strongest interaction and binding affinity to HSP-27 and TNF-a out of the selected proteins. The docking comparison of RA with standard drug also indicated RA forming more stable conformation with targeted proteins compared to TMZ on the basis of binding energy and protein ligand orientation.

Keywords: Rosmarinic Acid, GBM, Docking, HSP-27, TNF-a

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Effects of Rosmarinic Acid on Protein Expression and Glycosylation of Glioblastoma Multiforme U87 Cell Lines in Comparison with Temozolomide

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