INVESTIGATION THE EFFECTS OF COMBINED TREATMENT WITH TEMOZOLOMIDE AND GAP JUNTION INHIBITORS ON GLIOBLASTOMA

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INVESTIGATION THE EFFECTS OF COMBINED TREATMENT WITH TEMOZOLOMIDE AND GAP JUNTION INHIBITORS ON GLIOBLASTOMA

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ABSTRACT

INVESTIGATION THE EFFECTS OF COMBINED TREATMENT WITH TEMOZOLOMIDE AND GAP JUNCTION INHIBITORS ON GLIOBLASTOMA

Glioblastoma, a highly lethal brain cancer with a survival rate of 6.8%. Connexins are gap junction proteins that enable the cell-cell communication. They contribute to drug resistance as well as invasive and metastatic characteristics of cancerous cells. Specifically, Connexin 43 is the most abundant connexin protein in GBM cells which is shown to be involved in chemotherapy resistance. However, the mechanism of Connexin-43 mediated resistance is not revealed, yet. In this thesis, Connexin-43 inhibitors, 18β-GA and oleamide, are combined with Temozolomide (TMZ), a chemotherapeutic agent used in GBM, and the TMZ sensitivity is evaluated when Connexin-43 is inhibited. Also, the changes in the protein expression of drug resistance associated key members are investigated. It is demonstrated that TMZ sensitivity increases with Connexin-43 inhibition. However, when combined with TMZ, 18β-GA presents a more significant decrease in GBM cell viability compared to oleamide. Interestingly, protein expression profile indicates that chemoresistance related molecules are upregulated in combined treatment groups which requires new insights to Connexin-43 inhibition in GBM treatment.

ÖZET

TEMOZOLOMIDE VE BOŞLUK BAĞLANTILARI (GAP JUNCTION) İNHİBİTÖRLERİNİN KOMBİNE UYGULAMASININ GLİOBLASTOMADAKİ ETKİLERİNİN ARAŞTIRILMASI

Glioblastoma, 5 yıllık sağ kalım oranı %6,8 olan oldukça ölümcül bir beyin kanseridir Konneksinler, hücre-hücre iletişimini sağlayan boşluk bağlantı proteinleridir ve kanser hücrelerinin ilaç direnci ile invaziv ve metastatik özelliklerine katkıda bulunurlar. Spesifik olarak Connexin 43, kemoterapi direncine dahil olduğu gösterilen GBM hücrelerinde en bol bulunan konneksin proteinidir. Ancak, Connexin-43 aracılı direncin mekanizması henüz ortaya çıkmamıştır. Bu tezde, Connexin-43 inhibitörleri, 18β-GA ve oleamid, GBM'de kullanılan bir kemoterapötik ajan olan Temozolomid (TMZ) ile kombine edilmiş ve Connexin-43 inhibe edildiğinde TMZ duyarlılığı değerlendirilmiştir. Ayrıca, ilaç direnci ile ilişkili anahtar üyelerin protein ifadesindeki değişiklikler de analiz edimiştir. Connexin 43 inhibisyonu ile TMZ duyarlılığının arttığı gösterilmiştir. Bununla birlikte, TMZ ile birleştirildiğinde, 18β-GA, oleamide kıyasla hücre canlılığında daha önemli bir azalma sunduğu da ortaya konmuştur. İlaç direnci ilişkili moleküllerin kombine tedavi sonrası ifade seviyelerindeki değişimler analiz edildiğinde bu moleküllerin anlatımındaki artış Connexin-43 inhibitörlerinin çalışma mekanizmalarının ve bu inhibisyonun uzun vadeli etkileri üzerine çalışılması için bir gereklilik sunmuştur.

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18b-GA/18β-GA	18β - glycyrrhetinic acid
BBB	Blood Brain Barrier
Cas3,7	Caspase-3,7
CNS	Central Nervous System
Cx43/Cx-43	Connexin 43
GBM	Glioblastoma
TMZ	Temozolomide
WHO	World Health Organization

1. INTRODUCTION

1.1. CANCER: CLASSIFICATION, EPIDEMIOLOGY & STATISTICS

Cancer, a wide range of diseases defined by abnormal cell proliferation and invasive characteristics, is reported to be first or second leading reason of death in 112 out of 183 countries in 2019 by World Health Organization (WHO) [1].

Approximately, there are 200 different types of cancer which are classified according to tissue of origin (e.g. lung cancer) or the cell type they arised from [2]. Cell type origin dependent classification defines 5 main types; carcinoma, sarcoma, leukemia, lymphoma and myeloma and brain & CNS cancers [3,4]. Carcinomas originate from epithelial cells found on internal and external lining of bodily organs and horbours 80-90% of total cancer cases. Carcinomas subdivided as adenocarcinoma (arised from an organ or gland such as pancreatic cancer) and squamous cell carcinoma (SCC, arised from squamous epithelium such as SCC lung cancer). Sarcoma is originated from connective or supportive tissues like bones, fat and muscle (e.g. osteosarcoma). Leukemia, a liquid cancer, stem from blood-producing tissues as bone marrow and lymphatic system (e.g. acute lymphoblastic leukemia). In most cases, leukemia is associated with overproduction of malfunctioning white blood cells. Lymphomas, on the other side, are solid tumors that arise from nodes or glands of the lymphatic system. Lymphomas may also be originited in organs such as breast and called as extranodal lymphomas [5]. Otherwise, they subdivided into 2; Hodgkin and non-hodgkin lypmhoma and differentiated by the existence of Reed-Sternberg cells in the former [6]. Brain and CNS tumors may originate from glial cells (glioma), meninges (menengioma), neuroectodermal cells (medullablastoma), Schwann cells (shawannoma), or both from neuron and glial cells (gangliogliomas) [7].

Globally, incidence and mortality rate of cancer is enourmously increasing upon the life-time extension and increase of risk factor exposure. Accordingly to GLOBOCAN, 2020 statistics 19.3 million new cases and 10 million cancer-related deaths are reported globally. Asia accounts half of total cancer cases as well as the 58.3% of cancer-related deaths whereas Europe harbours 22.8%, and Americas' 20.9% of all cases. Top ten cancer types include female breast (11.7%), lung (11.4%), colorectal (9.4%), prostate (7.3%), non-melanoma of skin (6.2%), stomach (5.6%), liver (4.7%), cervix uteri (3.1%) and esophagus

(3.1%) cancers. Worldwide, estimated 28.4 new cancer cases are envisioned to occur by 2040, which is a 47% increase compared to 2020 [8]. Therefore, the need of global collobration regarding to introduction of new and effective therapautics to the use of cancer patients is growing even rapidly.

1.2. BRAIN & CENTRAL NERVOUS SYSTEM TUMORS

The central nervous system (CNS) embodies the brain and spinal cord which exerts sensory information receiving, processing and responsing functions. The brain, composed of nervous tissue, protected by the skull, cerebrospinal fluids and blood-brain barrier [9]. Blood-brain barrier (BBB), represented in Figure 1.1., a unique microvasculature system in which the vessels are continous and non-fenestrated, regulates the exchange of molecules, ions and cells between the blood and CNS. Therefore, BBB strictly protects the CNS homeostasis which is crucial for appropriate neuronal function and protection from extrinsic factors such as pathogens, toxins, and injury. The limited permeability of BBB is a major impediment for drug delivery which requires tremendous efforts to generate delivery systems that modulate and/or bypass the BBB for therapautic purposes [10].

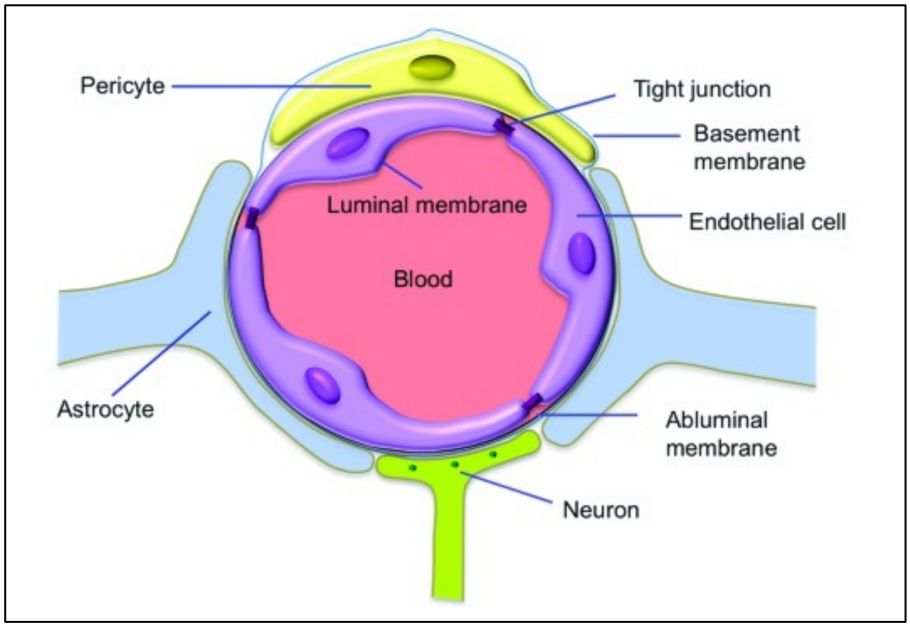

Figure 1.1. Representation of the blood brain barrier [11]

Anatomically, brain is divided into cerebral hemispheres and each hemisphere is divided into lobes: frontal, perietal, occipital and temporal [12], as seen in Figure 1.2..

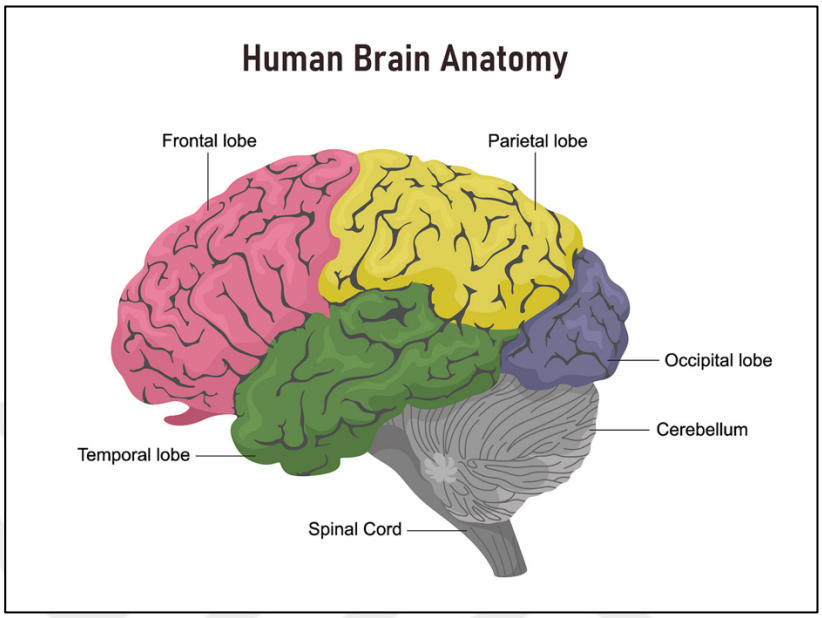

Figure 1.2. Representation of human brain anatomy [13]

Brain tumors can be found on lobes (supratentorial), cerebellum or brain stem (infratentorial). Independently of localization, brain tumors might cause headaches and seizures where location dependent symptoms might involve vision, speaking problems or tremors [14]. Currently, brain and CNS tumors constitues one of the cancer types that accounts for high morbidity and mortality rates. As seen in Figure 1.3., according to Surveillance, Epidemiology, and End Results (SEER) registrations 25.050 new cases (1.3% of all cancer cases) and 18.250 deaths (3% of all cancer-related deaths) are estimated for brain and CNS tumors in 2022 [15].

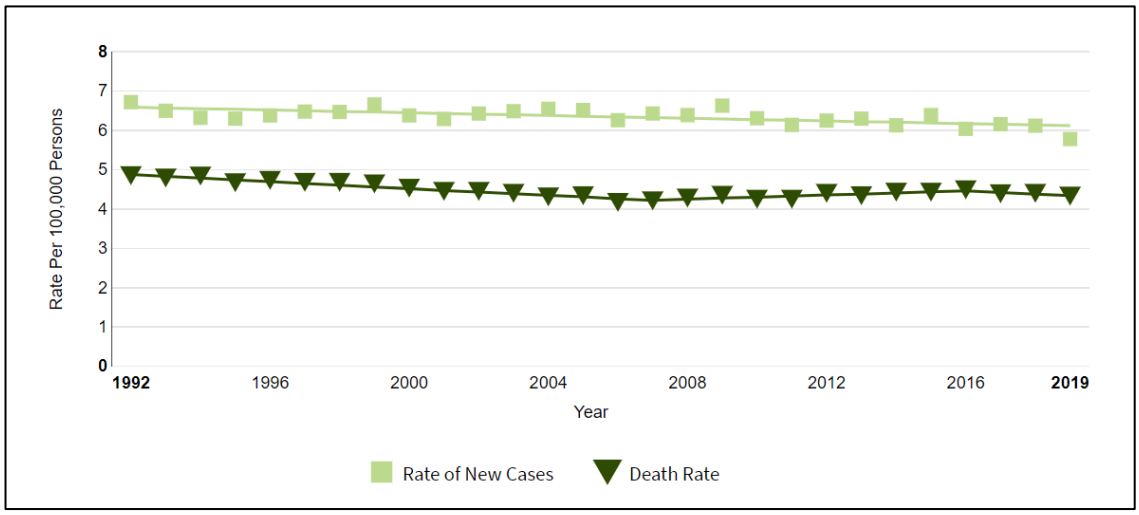

Figure 1.3. SEERs registration of new and death cases of Brain and CNS tumours according to years reveals that the new case and death rate of these tumours are steady demonstrating the lack of efficient preventive and therapeutical methods

Benign and malignant brain and CNS tumors are very diverse and distinct regarding to histological and clinical characteristics, treatment regimens, and prognosis. The etiology remains enigmatic. Though, the classification criteria has broadly changed and improved in recent years upon growing molecular knowledge, expanding expertise and technology in detection and diagnosis methods [16].

1.2.1. Benign Brain and CNS Tumors

Benign tumors have defined boundaries, slow growth rate and uncommon invasion of nearby healthy cells. Due to the physiological restrictions that they cause, benign tumors usually removed by surgery. Such benign tumors like colon polyps have a tendency to transform into malignant cells and require regular monitoring and surgical removal of the mass [17]. Pituitary tumors, meningiomas, and shwannoma are examples of benign brain tumors [18].

1.2.2. Malignant Brain and CNS Tumors

Malignant tumors, considered cancerous, exerts abnormal growth and have the ability to invade and metastasize to local and distant tissues. The metastatic spread occurs through the body via blood stream or the lymphatic system [17].

Brain and CNS tumors are distinct from any other neoplasm due to their specialized characteristic signature. In brain and CNS tumors distinct discrimination between malignant and benign characteristics is not explicit [19]. Anatomical localization may present a highly aggresive tumor phenotype regardless of the histological properties. Also, metastasis is a rare event in those tumors. However, the invasion across the brain parenchyma is common. Neuroblastoma, gliosarcoma, medullablastoma, and glioma are the examples of malignant brain cancers. However the aggressiveness may vary depending on the spesific mutations [19].

1.3. GLIOMAS

Gliomas, originated from glial, neuroglial stem or progenitor cells, comprise roughly 30% of all primary brain tumors and nearly 80% of all malignant brain tumors. The anatomic localization of gliomas determines the therapeutic approach and influences the prognosis. In total glioma cases, frontal lobe (40%), temporal (29%), parietal (14%) and occipital (3%) lobe localizations are seen. The gliomas may also be localized in deeper structures (14%) such as pituitary gland [20]. Gliomas defined in 3 common subtype based on the cell characteristics which are astrocytomas (arise from astrocytes), ependymomas (arise from ependymal cells), and oligodendrogliomas (arise from oligodendrocytes) [21]. Further classification is made as low-grade, atypical and, high-grade which determined by the mitotic activity, histological properties and distinct molecular signatures [22]. The least malignant form of gliomas is pilocytic astrocytomas while the most amalignant type is glioblastoma multiform (GBM) [21].

WHO grading system is the currently accepted international standard for the nomenclature and diagnosis of gliomas and divides gliomas into four grades from I to IV based on the degree of malignancy assessed by the histopathological criteria. While Grade I tumors are non-cancerous and slow growing tumors with lon-term survival capacity, Grade IV tumors

shows aggressive malignancy and grows rapidly. Grade II tumors are also slow growing tumors and might be cancerous or benign. Grade III tumors are malignant tumors and develop into higher grades with high frequency [23,24]. In case of a particular grading system for the cancer of interest is not available then the grading is done depending on the differentiation status in which grade I, well differentiated; grade II, mildly differentiated; grade III, poorly differentiated and grade IV, undifferentiated [25].

1.3.1. Glioblastoma Multiforme (GBM)

Glioblastoma (GBM) is one of the most aggressive central nervous system tumors. The survival rate of GBM, a rare tumor with a global incidence of less than 10 per 100000 people, is only about 14 to 15 months following diagnosis [26-28]. GBM is may be arised from neural stem cells (NSCs), astrocytes differentiated from NSC and oligodendrocyte precursor cells. Cellular origin and molecular signature have a role in tumorigenesis [29].

Oligodendrocytes and astrocytes give rise to this aggressive type of tumor. Glioblastoma is mainly found in frontal, parietal, occipital and temporal lobes, however this malignancy is rarely occurred in the brain stem, the cerebellum and the spinal cord [30]. For glioblastoma, different subtypes appear in the literature in order to classify it and these subtypes vary according to classification method. Identifying these subclasses plays a major role in successfully combating glioblastoma and improves the efficacy of targeted therapies [31]. Gene expression profiles, genetic alterations and DNA methylation status aid to establish these subclasses. According to gene expression profiles, GBM is classified into four subtypes contains proneural, neural, mesenchymal and classical. In proneural subtype of GBM, elevated PDGFRA gene expression and common IDH1 mutation are seen. Responding to chemotherapy and radiation traetment is nearly same with other three subtypes while survival rate of proneural subtype is higher [32]. Secondly, while comparing the gene expressions of normal brain tissue and neural subtype of GBM, similar patterns are observed. In addition, neural subtype tend to respond chemotherapy and radiation treatment better compared to other subtypes. In the classical subtype, mortality rate diminishes with intensive radiation therapy and chemotherapy. The last subtype categorized according to transcriptome-based data is mesenchymal subtype. Extreme necrosis and inflammation, tumor suppressor genes deletion such as PTEN and P53, angiogenesis and interstitial genes

overexpression are the features of the mesenchymal subtype of the glioblastoma. Poor prognosis is presented in mesenchymal subtype compared to other GBM subtypes even though responding intensive chemotherapy and radiation therapy [32].

Genomic investigations have considerably increased the comprehension of GBM by identifying the genetic alterations in oncogenes and tumor suppressor genes. Mutations which are occurred in IDH, PTEN and EGFR genes are particularly associated with survival rate of patients. Numerous researches indicate that IDH mutation status is highly valuable about prognosis and prediction of the GBM [33-34]. Isocitrate dehydrogenase (IDH) is a key enzyme participated in citric acid cycle and essential for resistance of oxidative stress. IDH1 is one of the IDH enzymes which is primarily located in the cytoplasm while IDH2 and IDH3 enzymes are found mainly in the mitochondrial matrix [35]. One of the study findings revealed that mutations may be occured in IDH1 gene after the low-grade glioma development and give rise to tumor progression to glioblastoma [36]. GBM is divided into two categories based on IDH mutation status which are IDH wild type and IDH mutation. Overall survival is higher in IDH mutant GBM patients and according to response of chemotherapy, IDH mutant GBM patients are better compared to IDH wild-type GBM patients [37]. A recent research demonstrated that IDH1 mutations result in the progression of low grade gliomas to Grade IV glioblastoma. It also established that the majority of glioblastomas and anaplastic astrocytomas with altered gene expression of IDH1 or IDH2 have a mutation in TP53 gene, but EGFR and PTEN gene mutations consists of only 3% of these cases. Contrarily, TP53 mutations are less common in glioblastomas and anaplastic astrocytomas, which have wild-type IDH1 and IDH2 genes, have more altered gene expressions in EGFR and PTEN with 74% of rate [36].

EGFR which is responsible for cell proliferation, development and differentiation is another investigated gene for GBM classification. EGFR amplification commonly coexists with EGFR mutation. EGFRvIII, mostly mutated EGFR activates mitotic and anti-apoptotic signaling pathways and play a role in sustaining these pathways, accordingly tumorigenic potential of GBM is improved [38].

PTEN which is a protein producing PIP2 (phosphatidylinositol-4,5-bisphosphate) following the 3’ phosphorylation of the inositol ring in PIP3 (phosphatidylinositol-3,4,5-trisphosphate) dephosphorylation have a crucial role in the AKT signaling pathway inhibition [39]. Normally, in quescent and differentiated cells, the PI3K/AKT pathway is inactive, however,

activation of this pathway induces cancer by modulating the cell cycle. The crucial role of PTEN arises in this point because their deficiency leads to PI3K/AKT pathway activation, consequently it resulted with more aggressive phenotypes of GBM [40].

Subtypes of GBM may also be characterized based on DNA methylation status which is caused by the epigenetic changes. DNA methylation offers biomarkers for detection of cancer earlier and prognosis of the cancer, as well as provides novel techniques for clinical applications. For instance, worse survival rates are significantly correlated with MGMT promoter methylation, which is a prognostic factor for patients who suffer from glioblastoma [41]. MGMT (O6-Methylguanine-DNA Methyltransferase) is a DNA repair enzyme that removes genotoxic O6-meG (O6-methylguanine) adducts caused by the temozolamide (TMZ). Therefore, MGMT promoter methylation that leads to silencing of MGMT gene, is crucial for preventing DNA repair and causing genotoxicity to GBM [42-44]. A more comprehensive genome and expression profile is required due to variability of GBM in order to find out the possible responsiveness of therapeutic approaches. At this point, DNA methylation may contribute comprehending glioblastoma and determining a therapeutic approach. Therefore, additional molecular biomarkers and identified new therapy targets is offered by DNA methylation-based subtypes. Collecting these classifications based on transctiptome, genetic alterations and DNA methylation with together, provides more extensive classification for GBM [31].

1.3.1.1. Other GBM-associated Pathways

JAK/STAT pathway is also crucial in glioblastoma. JAK/STAT pathway which consists of Janus protein kinases (JAKs) and signal transducer and activator of transcription proteins (STATs), activated by binding of the ligand to the various receptors found on the cell membrane and starts the phosphorylation of JAKs following conformational change of receptors by the aid of JAKs, and then phosphorylation of STAT proteins [45]. Phosphorylated STAT monomers are activated and dissociated from their docking sites for triggering transcription of various distinct genes by interacting with particular DNA-binding sites following migrating to the nucleus. After binding of the ligand to a cytokine and growth factor receptors found on the membrane, JAK1, one of the members of the JAK family proteins, is activated [46]. A number of cancer cell lines and human tumors comprising solid tumors and blood malignancies is identified to activate STATs, particularly STAT3 [47]. Tyrosine kinase signals lead to translocation of STAT3 to nucleus in order to regulate gene

expressions by interacting with components within promoters, after the formation of STAT3 dimers. The regulation of several pathways that are crucial for the development of tumors including apoptosis, progression of cell cycle, metastasis and invasion, angiogenesis of tumors and evasion of the tumor cell from the immune system. In gliomas, a research based on histopathological staining is revealed that JAK1, phosphorylated JAK1 and STAT3 are immunohistochemically determined as positively stained. This determination indicates that, patients with glioma showed a substantial correlation between the JAK1 ans STAT3 expression and prognosis, grades and KPS scores [48]. Another study about STAT3 activation indicates that tissues belong to GBM patients contain higher activation of STAT3 in comparison to control tissues [49]. Expression of phosphorylated STAT3 (pSTAT3) is related with poor survival in patients who suffer from glioblastoma and its expression also related with immune infltration [50]. Additionally, it is shown that STAT3 is a powerful regulator of responding inflammation and it affects this response negatively by suppressing activation of macrophages [51].

Wnt/β-catenin signalling is another mechanism that affects glioblastoma in terms of proliferation of the glioma cells and their invasion. In GBM, due to the significant correlation between malignancy and elevated levels of protein and mRNA in GBM and higher grade astrocytomas, β-catenin is considered as a prognostic marker [52]. Various developmental processes, containing nervous system's neurogenesis, Wnt signals play a crucial role [53]. It is suggested that canonical Wnt/ β catenin signal transduction pathways affects proliferation of neural progenitor cells (NPCs) and their decisions about lineage variably [54]. In proliferating neural stem cells, expression of Wnt5a, which is the molecule of noncanonical Wnt pathway that is independent from β-catenin molecule, is observed and during differentiation, expression level of Wnt5a rises [55]. Wnt genes, including *Wnt2, Wnt5a* and *Wnt7a*, are linked to a numerous cancers. For instance, in high grade gliomas, noncanonical Wnt5a expression is elevated and cell proliferation is promoted by Wnt5a. Compared to normal tissue samples, Wnt5a expression is higher in GBM, therefore it is suggested that there might be an association between Wnt5a and tumor progression [56].

1.4. CELL-CELL COMMUNICATIONS

Multicellular systems require cell-to-cell communication due to convenient function and development [57]. Epithelial cells are connected with each other by alongside junctions that their protein composition and functions are varied. Extracellular regions of the sticky transmembrane proteins that attach these junctions connect with neighboring cells via similar proteins found on the cell. Protein complexes of each cell link the cytoskeleton and the adhesive transmembrane proteins, accordingly mechanical support of cells and tissues are provided. Additionally, junction proteins provide that positional information is conveyed to the nucleus by via recruiting intracellular signalling molecule. A variety of specialized junctions are found on the lateral side of epithelial cells. One of these specialized connections is tight junctions which constitute a strong barrier between cells and nothing, including ions, can flow through. Other specialized protein junctions are gap junctions and these junctions play a role in cellular communication in several tissue types. In contrary to tight junctions, a narrow pore is provided between neighboring cells by gap junctions and ions even small molecules have a permisson to pass from one cell to another through these pores. Tissue homeostasis, which means equilibrium within a tissue, is provided by gap junctions through diffusion. For the tissue function, regulation of gap junctions become more critical. In this regulation, adhesive molecules such as integrins take an active role in order to protect favorable structure of tissue [58].

Gap junctions, shown in Figure 1.4., which are collections of intercellular channels, are responsible for the diffusion of small molecules and ions between adjacent cells directly [59]. Docking of the hexameric structures, which are called connexons constituted from six connexin molecules, create an axial channels, accordingly an extraxellular gap is formed [60]. Multiple physiological functions, including the embryonic development, proliferation and differentiation of cells, electrical coupling between cells, uterus and heart contraction, depend on intercellular communication via gap junctions [59]. For instance, incredible amount of gap junctions in cardiac tissue contribute to the ability of tissue beating rhytmically with the rapid flow of ions via these junctions [61]. In response to metabolic stimulation, gap junctions might open or close and that enables for long distance intercellular communication as well as glioblastoma cells to provide paths for microinvasion of brain [62].

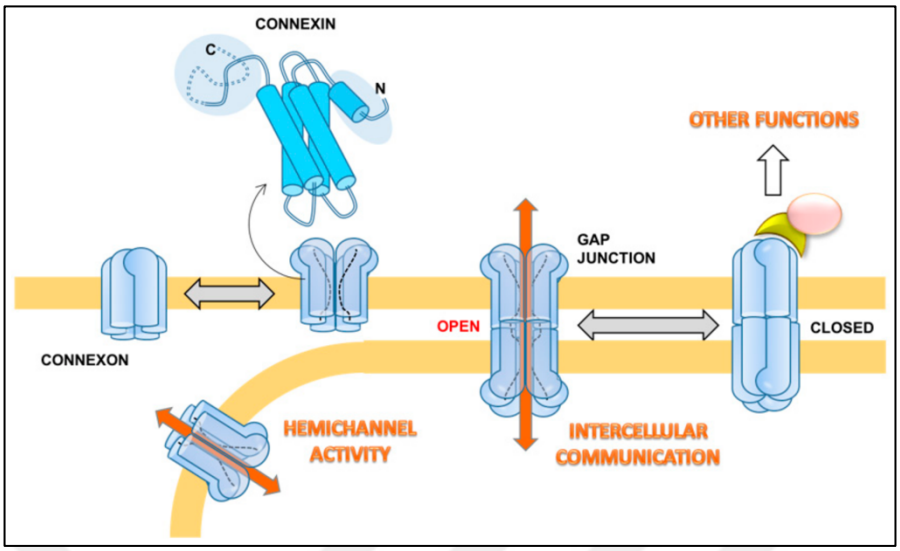

Figure 1.4. Schematic representation of gap junctions [63]

1.5. CONNEXIN-43

Connexin, which facilitates cellular communication, is a unique form of transmembrane proteins [64]. Structurally, one cytoplasmic loop, two extracellular loops and one cytoplasmic N-terminal and C-terminal regions gather to form connexin subunit. Despite sharing similar structure in general, each of the connexin isoforms have different amino acid sequences. Connexins are encodded by 21 genes in humans and, in terms of their molecular weights, names of connexins are determined [65-66]. The main connexin member is Connexin 43, with 43 kD molecular weight, are expressed in astrocytes and a subset of cells in nearly all GBM tumors. There is an inverse relatioship between Connexin-43 expression and glioma grades. Therefore, in high grade gliomas, Connexin-43 expression diminishes [67]. The progression of gliomas is assisted by Cx43 downregulation as well as downregulation of Cx43 prevents infiltration through Cx43 [59]. However, newly emerged researches indicate that tumor invasion is promoted by gap junctions, especially Cx43, between astrocytes and glioblastoma. Identifying functional gap junctions between astrocytes and GBM cells is crucial for comprehending invasion [59]. Cx43-mediated communication between astrocytes and glioblastoma at the tumor boundary is encouraged the invasion. Additionally, miRNA transfer via Cx43 also manipulates astrocytes for permitting invasion [68]. Also, it is demonstrated that Connexin-43 drives the Temozolamide resistance by PI3K activation [69]. It is widely accepted that Connexin-43

regulates in part of tumor progression, metastasis and chemoresistance in GBM [70]. Exchanging metabolites, ions, secondary messengers and solutes, depends on gap junctions, are required for survival of the cells, including cancer cells. Transfering of these essential substances can be prevented by blocking gap junctions, then this blokage induces cellular apoptosis [71,72]. Different compounds that interrupt communication via gap junctions are known as gap junction blockers (GJBs). Carbenoxolone, oleamide, glycyrrhetinic acid, quinidine, quinine, heptanol, octanol, mefloquine, fenamates, anandamide, retinoic acid, sodium propionate, spermine, anesthetics, 2-aminoethoxydiphenyl borate (2-APB), aminosulfonates are the inhibitors of gap junctions that block the cell to cell communication. Especially, carbenoxolone, quinidine, quinine and mefloquine are the gap junction inhibitors used in medicine as a drug and their side effects have been identified on human [73]. Glycyrrhetinic acid (GA) is a triterpenoid, which are plant-based substances, derived from Licorice roots (Glycyrrhiza glabra). In various cell lines of cancer, glycyrrhetinic acid is demonstrated to have antitumor effects, as well as antiproliferative effects, mediated by the stimulation of apoptosis [74]. Strong correlation between using glycyrrhetinic acid as a chemical for treatment and activation of caspases [75]. Glycyrrhetinic acid, which has two isoforms alpha and beta, is pharmacologically safe for human due to being comparatively non-toxic in healthy cells, accordingly using of this gap junction inhibitor take an advantage for the cancer treatment [76]. Oleamide, which is derived from cerebrospinal fluid belongs to sleep-deprived cats, is the member of primary fatty acide amides (PFAMs) and demonstrated to have antiproliferative and non invasive properties. Chemotherapy is enhanced by using this sleep-inducing gap junction inhibitor [77]. One of the differences between glycyrrhetinic acid and oleamide is that oleamide does not affect the transmission of calcium wave stimulation, but glycyrrhetinic acid affects both gap junction communiction and intercellular calcium signaling [78]. Another difference between these two gap junction inhibitors is their target connexin varieties. Apart from these differences, gap junction inhibitors meet on a common ground by blocking the Connexin-43 [79,80].

1.6. TEMOZOLOMIDE (TMZ) IN GBM TREATMENT

Temozolomide (TMZ), is a member of a broader group of DNA alkylating compounds, generally utilized as the standard chemotherapeutic agent for diagnosed GBM patients. The discovery of antitumorigenic effects of TMZ was done in the 1940s, but original FDA

approval came in 2005. The addition of TMZ as a chemotherapy to radiation therapy, median overall survival of patients increases approximately two months [81,82]. For GBM survival, one of the most crucial improvements is using TMZ as a chemotherapeutic agent. Particular locations on single strand DNA are affected by TMZ. This alkylating agent leads to DNA methylation at the O6 position of guanine, N3 position of adenine and N7 position of guanine, accordingly O6 site alkylation on guanine causes the O6-methylguanine adducts formation and ends up with the addition of thymine residues rather than cytosine. Apoptosis and cell cycle arrest are caused by these irreparable mutations which create the double-stranded and single-stranded breaks on DNA. A new approach about GBM classification, provided by World Health Organization (WHO) in 2016, molecular features of tumor, in addition to histological analysis, is might be useful for combining with GBM diagnosis and treatment. In GBM, the status of the enzymes IDH and MGMT is particularly significant. For instance, there is a strong association between better prognosis, and methylated MGMT and mutant IDH gene [83]. Nevertheless, aproximately 10% of tumors are IDH-mutant and MGMT methylation is observed in nearly 50% of tumors, and during treatment, MGMT methylation status might be altered [42,84,85]. Molecular classification of tumors provides to comprehend responses of tumor subtypes to TMZ treatment. For instance, while having a worse prognosis, wild-type IDH tumors may respond to TMZ better [83]. Comprehending of mechanisms of resistance to TMZ have an importance in the enhancement of GBM treatment and also classification [81].

1.6.1. Chemoresistance in TMZ

Even TMZ is widely used in GBM treatment, plays a crucial role in tumor recurrence and resistance. Signaling pathways, Wnt/β-catenin and JAK/STAT pathways play a role in TMZ resistance, accordingly abnormal signaling in these pathways lead to chemoresistance and GBM development [81]. Wnt3a, which is a ligand of β-catenin-dependent Wnt pathway, is overexpressed in GBM, and induce TMZ resistance and stemness [86]. Therefore, canonical pathway activation has an effect on glioma stem cells (GSCs) development [87]. Not only canonical pathway, abnormal activation of β-catenin-independent pathway also affects GBM tumorigenicity. GSCs have higher Wnt signaling activity compared to normal stem cells [88]. Epithelial-mesenchymal transition (EMT) is related to spreading of tumors and TMZ resistance in GBM, and this process is influenced by Wnt signaling pathway [89,90]. Wnt5a

promote progression of tumor and chemoresistance due to encouraging EMT and self renewal of GSCs [91]. In addition to Wnt pathway, stemness of glial cells stimulation is also related with JAK/STAT pathway and STAT3 activation influence the change in proneural subtype to mesenchymal subtype of GBM [92]. Elevation of MGMT expression and STAT3 overexpression are related with each other, accordingly lead to TMZ resistance in GBM [93]. Understanding the mechanism of TMZ resistance in GBM is vital to create future treatment methods.

1.7. APOPTOSIS

Apoptosis, which is an energy-dependent programmed cell death, is a natural phenomenon that arises throughout aging and development as well as keeping cell populations in a balance in tissues. In addition to providing homeostasis, apoptosis occur for responding immunological reactions or diseases and toxic chemicals, which destroy the cells [94]. For instance, in cancer therapy, chemotherapeuticals and radiation lead to DNA damage, accordingly apoptotic cell death is triggered via a p53-dependent mechanism. Apoptosis is influenced by a variety of stimuli and circumstances, however not all cells may be killed by a given stimulation. While some cells are affected by the hormones, that may induce apoptosis, other cells may not be affected. The morphology of the cells are altered in apoptosis, shrinkage of the cell lead to smaller cell, condensed cytoplasm and closely packed organelles. Shrinkage of the cell and chromatin condensation, that is called pyknosis and known as the typical feature of apoptosis, can be visualized by light microscopy during early apoptotic phase [95,96]. Morphological changes in apoptotic cells lead to fragmentation of apoptotic cells into apoptotic bodies, a subset of apoptotic extracellular vesicles [97]. These apoptotic bodies encompass packed organelles, their integrity is still preserved, and cytoplasm. Parenchymal cells, macrophages and neoplastic cells are help to phagocytosis of these apoptotic bodies. Cells, which undergoes apoptosis, do not release their cellular components into the neighboring cells and phagocytosed quickly by the engulfing cells, which do not generate any anti-inflammatory response, accordingly inflammatory reaction do not be observed in apoptosis [98]. The perfect mechanism of apoptosis distinguishes it from necrosis, known as an energy-independent toxic process rely on uncontrolled release of cellular components into the neighboring cells. As it is understood, a complicated collection of molecular activities that depend on energy comprise the mechanisms of

apoptosis. According to researches that are conducted discuss this mechanism into two main pathways, which are extrinsic and intrinsic. Extrinsic pathway relies on the death receptors, while intrinsic pathway depends on mitochondrial activity. However, these pathways are connected to each other and one pathway can be influenced by the molecules belong to other pathway [99]. In addition to these pathways, perforin/granzyme B pathway, which is T-cell mediated, also affects apoptosis. Granzyme A or granzyme B participate in this pathway for triggering apoptosis. Three of these apoptotic mechanisms meet at the same point of the execution pathway, that is triggered by Caspase-3 cleavage, and leads to apoptotic body formation, following several cascades. Differently, granzyme A pathway uses single stranded DNA damage for triggering cell death, which do not depend on caspases [100]. Caspases are extensively expressed as inactive proenzymes, and other procaspases are activated by these proenzymes, subsequently they turn into their active form. This leads to starting of protease cascade. Caspases cleave aspartic acid residues of proteins and is an irreversible route towards cell death when caspases are activated. Caspases can be classified into three groups which are initiator caspases, including caspase-2, caspase-8, caspase-9 and caspase-10, executioner caspases, also known as effectors including caspase-3, caspase-6 and caspase-7, and inflammatory caspases, consist of caspase-1, caspase-4 and caspase-5 [101]. Apart from these ten major caspases, there are also another types of caspases which are caspase-11, caspase-12, caspase-13, which is not found in human, and caspase-14, which is not expressed in in adult tissues. During septic shock, regulation of apoptosis and maturation of cytokines are done by caspase-11, while caspase-12 which is localized in endoplasmic reticulum (ER) is responsible for apoptosis which is specific for ER [102]. Transmembrane receptor-mediated interactions, involving death receptors, tumor necrosis factor (TNF) gene family receptors, are the components of the extrinsic signaling pathways triggers apoptosis, while receptor-independent stimuli, which affects their targets directly and initiates mitochondrial events, start apoptosis in the intrinsic signaling pathways [103]. In death receptor-mediated apoptosis, activated caspase-8 plays an active role for initiating apoptotic cascades by triggering execution phase of apoptotic cell death. In intrinsic pathway, stimulation by several stimuli, including hormones, growth factors, cytokines, toxins, radiation and hypoxia, leads to mitochondrial transmembrane potential loss, following mitochondrial permeability transition (MPT) pores opening due to the inner membrane alterations in mitochondria. Then, pro-apoptotic proteins are released through these pores into the cytosol and caspase-dependent pathway is triggered [104]. Caspase-9 is

activated, following apoptosome formation caused by the cytochrome release from the mitochondrial pores, and execution pathway is triggered [105]. As mentioned before, execution pathway is the meeting point of the other apoptotic pathways. Cytoplasmic endonuclease, which is responsible for nuclear material degradation, and proteases, that plays a role in cytoskeletal and nuclear proteins degradation, are activated by execution caspases. Effector caspases, including caspase-3, caspase-6 and caspase-7, lead to biochemical and morphological changes in apoptotic cells, following cleavage cascade [106]. As a result, apoptotic bodies are formed and phagocytosed by related cells without harming neighboring cells.

Newly emerged studies have demonstrated that combining TMZ with other molecules, increase the efficiency of TMZ. Combining TMZ and radiotherapy with Lomustine, which is another DNA alkylating agent, increases survival rate of patients from nearly 31 months to 48 months and it is undergoing phase 3 studies currently [107]. Afatinib, which is ERBB2 inhibitor and targets epidermal growth factor receptor (EGFR), combined with TMZ for GBM treatment and proliferation of cancer cells decreases due to prevention of migration, invasion and survival of the cells by blocking EGFRvIII/Akt signaling [108].

In this thesis, it is proposed to investigate the effects of gap junction inhibitors, which are oleamide and 18β-GA, combined with TMZ on glioblastoma.

2. MATERIALS AND METHODS

Materials and methods used in this study are shown below.

2.1. REAGENTS

18β-Glycyrrhetinic acid (18β-GA) (Sigma-Aldrich; #G10105) and oleamide (Sigma-Aldrich; #02136) were dissolved in DMSO and final concentration of solutions was 25 uM.

2.2. CELL CULTURE CONDITIONS

The A172 and U87 cell lines are used as GBM cell lines and maintained in Dulbecco's modified Eagle's medium (DMEM) contained 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (PenStrep). Immortalized Human Astrocytes, which was immortalized by SV40 and purchased from abm, is used as a healthy cell line and maintained in Prigrow IV medium contained GlutaMax, 10% FBS, 1% penstrep and 0,01% epidermal growth factor (EGF). For the Immortalized Human Astrocytes cells, collagen coated flask is used. In the tissue culture process, cells were maintained at 37^{o}C in an incubator which contains 5% CO_2 and 95% air. The cells were subcultured when they reached at approximately 80% of intensity by the aid of 0,25% trypsin. After applying trypsin, cells were incubated at 37^{o}C for 2-3 minutes for detaching cells from the surface of the flasks. Then, cells were centrifuged, following stopping the trpsin activity with their FBS containing mediums. According to their recommended culture conditions, GBM cells were centrifuged at 1200 rpm for 5 minutes while Immortalized Human Astrocytes cells were centrifuged at 1500 rpm for 3 minutes. After centrifuge step, cells were resuspended with their mediums and cultured in new flasks.

2.3. STABLE TRANSFECTION OF CELLS

Astrocyte and GBM cells were stably transfected by pLenti CMV GFP puro plasmid (Cat. No: #17448, Addgene, USA) and CMV-dsRED respectively. Lentiviral plasmids packaged by using HEK293T cell line. Briefly, 4x10^5 HEK293T cells were seeded in 10 cm^2 petri

dish. Next day, HEK293T cells starved for 2 hour with Opti-MEM. After starvation, medium is exchanged to the complete cell culture media. Packaging plasmids pCMV dr8.2 VPR (Cat. No: #8455, Addgene, USA), pCMV-VSV-G (Cat. No: #8454, Addgene, USA) and pLenti CMV GFP puro or CMV-dsRED combined in 1: 0.1 :1 ratio within opti-MEM, respectively. Totally, 12 ug DNA is used. FuGene HD (Cat. No: E2311, Promega, USA) is added to the DNA mixture as instructed by the manufacturer and incubated for 20 minutes, after then the mixture is added on to the HEK293T cells. At 48 and 72nd hours, cell culture media is collected and filtered through 0.45 um filters and viral supernatant were stored at -80^{o}C until use.

Astrocytes and GBM cells seeded in 6-well plates as 4x10^4 cells. Before the lentiviral transduction cells were starved for 2 hours by opti-MEM. Then, medium is removed and 1 mL of viral supernatant is introduced to the cells with 6 ug/mL (for astrocytes) and 8 ug/mL (for GBM cell lines) polybrene and centrifuged at 12000 RPM for 2 hours. Following the centrifugation step, viral supernatant is removed and fresh viral supernant is introduced to the cells by diluting in 1:2 ratio with normal cell culture media. After 24 hours, media is exchanged and cells were checked for their GFP and dsRED fluorescence in flow cytometry (BD,FACSCalibur, BD Biosciences, San Jose, CA).

2.4. CELL VIABILITY ASSAY USING MTS

For the assesment of viable cells in terms of their cytotoxicity and proliferation, The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, a colorimatric method, was used. The mechanism of this assay depends on 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, which is a tetrazolium compound, and this compound reduced to formazan compound in the presence of NADH and NADPH by the viable cells. Briefly, each of the cells were plated on 96-well plates with 3 repetition for 24h, 48h, 72h and 96h incubations. For determining appropriate dosage of the TMZ and gap junction inhibitors (oleamide and 18β-GA), cells were incubated with different concentrations of each drugs, separately. Concentration range of the TMZ and chemicals were determined according to literature [109-112]. The dosage interval of TMZ is between 100 uM and 300 uM (100 uM, 150 uM, 200 uM, 250 uM and 300 uM). For the gap junctions, doses of oleamide were determined between 5 uM to 25 uM (5 uM, 10 uM,

15 uM, 20 uM and 25 uM) and 18β-GA doses were determined from 50 uM to 200 uM (50 uM, 75 uM, 100 uM, 150 uM and 200 uM). DMSO was used as a control for gap junction inhibitors due to dissolving oleamide and 18β-GA in DMSO and dilution were done with cell culture mediums belongs to cells. Immortalized Human Astrocytes and GBM cell lines (A172 and U87) were treated with oleamide, 18β-GA and TMZ, separately. Every 24 hours for 4 days, 20% MTS containing cell culture mediums were added in each well and incubated until the color of medium containing 20% MTS turned into reddish color, it approximately took 45 minutes, then they were transferred into a new 96-well plate for reading their absorbance based on formazan quantitiy. The plate were put in the microplate reader and their absorbance was measured between the 490 nm and 660 nm wavelengths. Cytotoxicity of drug dosages were determined by calculating the percentages of viable cells, compared to untreated cell groups.

2.5. CELL VIABILITY OF CO-CULTURED CELLS BY FLOW CYTOMETRY

Stable transfected eGFP astrocytes cells and dsRed A172 cells were co-cultured in 1:1 ratio on collagen-coated 6-well plates. Immortalized Human Astrocytes cells were green, while GBM cell lines were red. The co-culture experiment groups included control group with DMSO and oleamide group for each of the GBM cell line co-culture with astrocytes. Cells were incubated for 24-48-72-96h, and after incubation, cells were collected from the cell culture plates according to cell culture methods mentioned in Material and Method part in 2.2 section. After cells were centrifuged, an additional centrifuge was done with PBS in order to wash them and remove from medium. Then, cells were resuspended with DAPI in PBS and analyzed by flow cytometry. Then, same experiments were done with the gap junction inhibitor, with 25 uM concentration of oleamide, in order to determine its effect on cell to cell communication.

2.6. PROTEIN EXPRESSION STUDIES BY WESTERN BLOT

GBM cells and Immortalized Human Astrocytes cells were incubated with appropriate concentrations of oleamide, 18β-GA and TMZ for 24h. Protein levels of Connexin-43, apoptosis-related markers, which are Caspase-3, Cleaved Caspase-3, Caspase-7 and Cleaved

Caspase-7, and GBM-associated pathway members, including JAK1, STAT3, pSTAT3, Wnt5a were analyzed by using western blot. Apoptosis assay was performed by using Caspase-3, Caspase-7 and their active forms, which are cleaved Caspase-3 and cleaved Caspase-7 primers. These caspases were selected due to their executioner role [90,116]. GAPDH with 1:5000 ratio is used as a housekeeping gene. After handling cells, according to cell culture methods mentioned in 2.2., protein quantification was done by using BCA Assay. BCA (Bicinchoninic acid) assay, a colorimetric assay, depends on the cuprous ion production. Under alkaline conditions, cuprous ion is produced by the biuret reaction, and the reaction of this ion and sodium salt of bicinchoninic acid forms bicinchoninic acid cuprous complex that gives deep blue color and read at 562 nm at ELISA reader [113]. According to BCA results of cells, cells were prepared for the Western Blot analysis so that all cells were at the same concentration, which was 50 ug. Dilution of the proteins were done by RIPA buffer containing 1% phosphatase inhibitor. The prepared samples were incubated at 95^{o}C for 5 minutes, then samples, also marker, were loaded into the wells of SDS-PAGE gel. In order to facilitate the protein separation, SDS-PAGE gel was prepared with two different densities. Upper part of the gel, that contains wells for sample loading, contained 8% SDS-PAGE concentration, while the density of SDS-PAGE in lower part of the gel was at 12%. After running of the samples on the gel, they were transferred to a PVDF membrane. For blocking, 5% non-fat milk powder was dissolved in TBS-T and PVDF membrane were incubated with this blocking solution on the shaker at room temperature for 1 hour. Then, primary antibodies; GAPDH (Cell Signaling Technology, Inc; 2118; 1:5000), Anti-Cx43 (abcam; ab11370; 1:1000), JAK1 (Santa Cruz Biotechnology, Inc; sc-1677; 1:3000), STAT3 (Santa Cruz Biotechnology, Inc; sc-293151; 1:1000), pSTAT3 (Santa Cruz Biotechnology, Inc; sc-136193; 1:3000), Wnt5a (Sigma-Aldrich; MABD136; 1:1000), Cas3 (Cell Signaling Technology, Inc; #14220; 1:1000), Cleaved Cas3 (Cell Signaling Technology, Inc; #9664; 1:1000), Cas7 (Cell Signaling Technology, Inc; #12827; 1:1000), Cleaved Cas7 (Cell Signaling Technology, Inc; #8438; 1:1000) were prepared according to their reccommended concentrations by using non-fat milk powder in TBS-T, and incubated at 4C overnight. Secondary antibodies, that were anti-rabbit and anti-mouse and used according to source of primary antibody, were prepared with 1:3000 dilution by using non-fat milk powder in TBS-T. Then, PVDF membrane was incubated for 1,5 hours with the secondary antibody. For the imaging of the PVDF membrane, enhanced chemoluminescence solution (ECL) was added on the membrane, following cleansing the membrane from secondary antibody, and

incubated for 3 to 5 minutes in dark. Chemoluminescence were detected based on luminol radiance and images of the gels were analyzed by using Image J.JS software.

2.7. STATISTICS

Statistics of MTS results and Western Blot results were analyzed by using GraphPad Prism 9.4.1 software. Ordinary one-way ANOVA test was used for analysis; **** $P \leq 0{,}0001$, *** $P \leq 0{,}001$, ** $P \leq 0{,}01$, * $p \leq 0{,}05$, ns $p>0{,}05$.

3. RESULTS

The results of cell viability, flow cytometry and western blot analysis were presented in detail below.

3.1. DETERMINATION OF APPROPRIATE DOSES OF TMZ AND GAP JUNCTION INHIBITORS

Figure 3.1. indicates cell viability of Immortalized Human Astrocytes, while cell viability of GBM cell lines were shown in Figure 3.2.. Treatment with TMZ between 24h and 96h incubation indicated that 150 uM of TMZ had an effect on A172 cells. However, the appropriate dosage for TMZ affected to Immortalized Astrocytes cells with a minimum damage. In U87 cells, cell viability was slightly decreased, so significant data could not be taken.

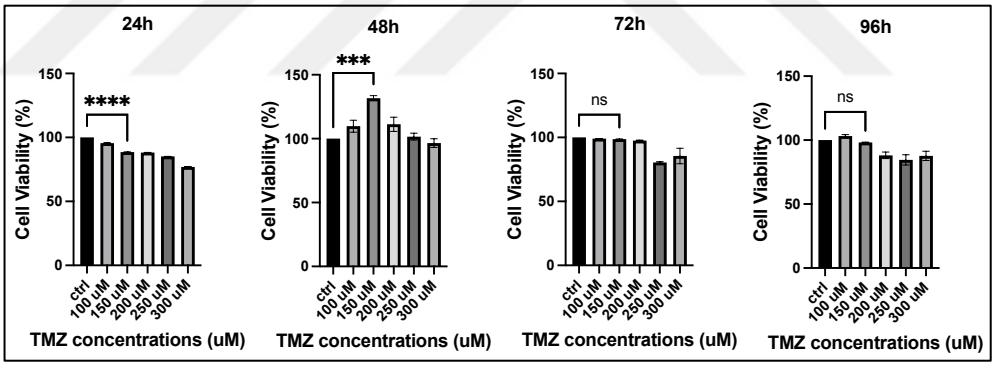

Figure 3.1. Cell viability of Immortalized Human Astrocytes cells which were treated with TMZ with the concentration range of 100 uM to 300 uM for 24h, 48h, 72h, 96h incubation.

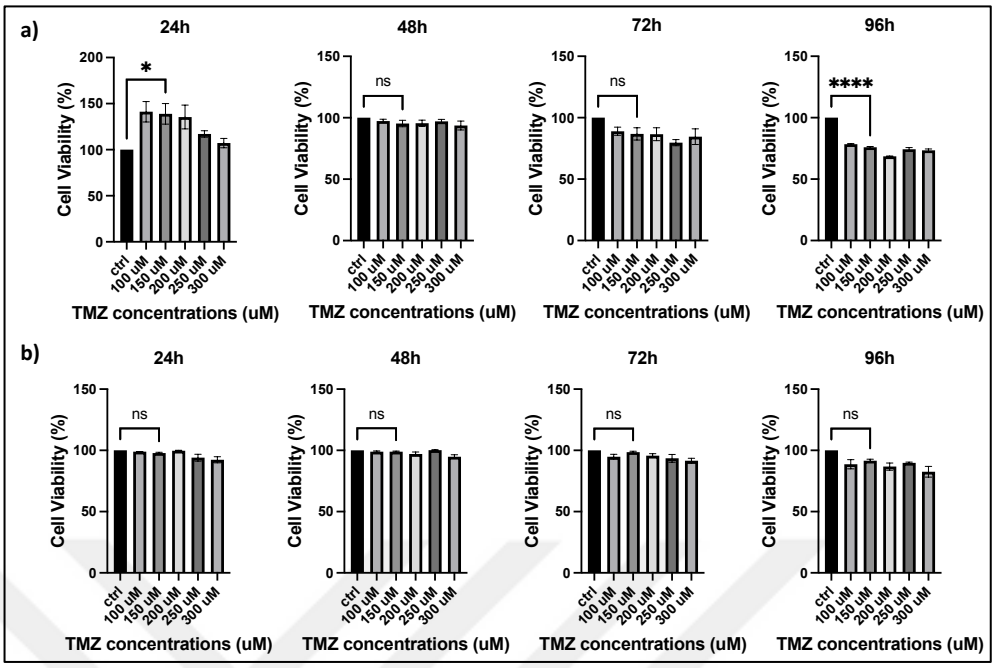

Figure 3.2. Cell viability of a) A172 and b) U87 cells which were treated with TMZ with the concentration range of 100 uM to 300 uM for 24h, 48h, 72h, 96h incubation.

After determination of the appropriate dosage of TMZ, combined treatment with TMZ were done in order to indicate the effect of gap junction inhibitors in the presence of TMZ on GBM cell lines and astrocytes. Cell viability assay was performed with the gap junction inhibitors only and their combined form with TMZ with their range of dose concentrations by keeping constant in TMZ concentration.

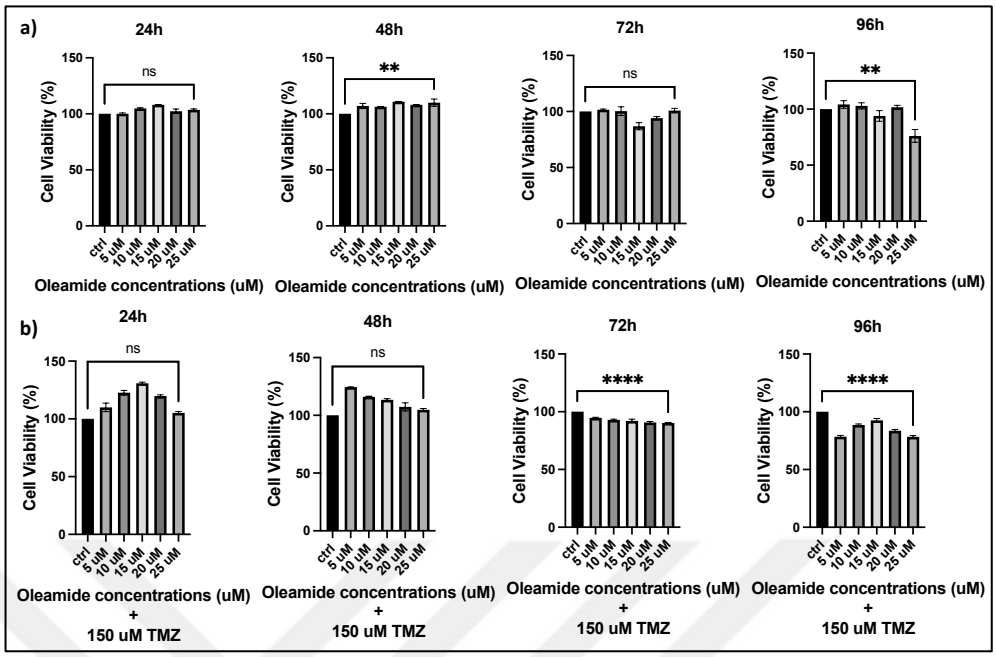

Figure 3.3. Cell viability of Immortalized Human Astrocytes cells which were treated with a) oleamide with the concentration range of 5 uM to 25 uM and b) oleamide + 150 uM of TMZ for 24h, 48h, 72h, 96h incubation.

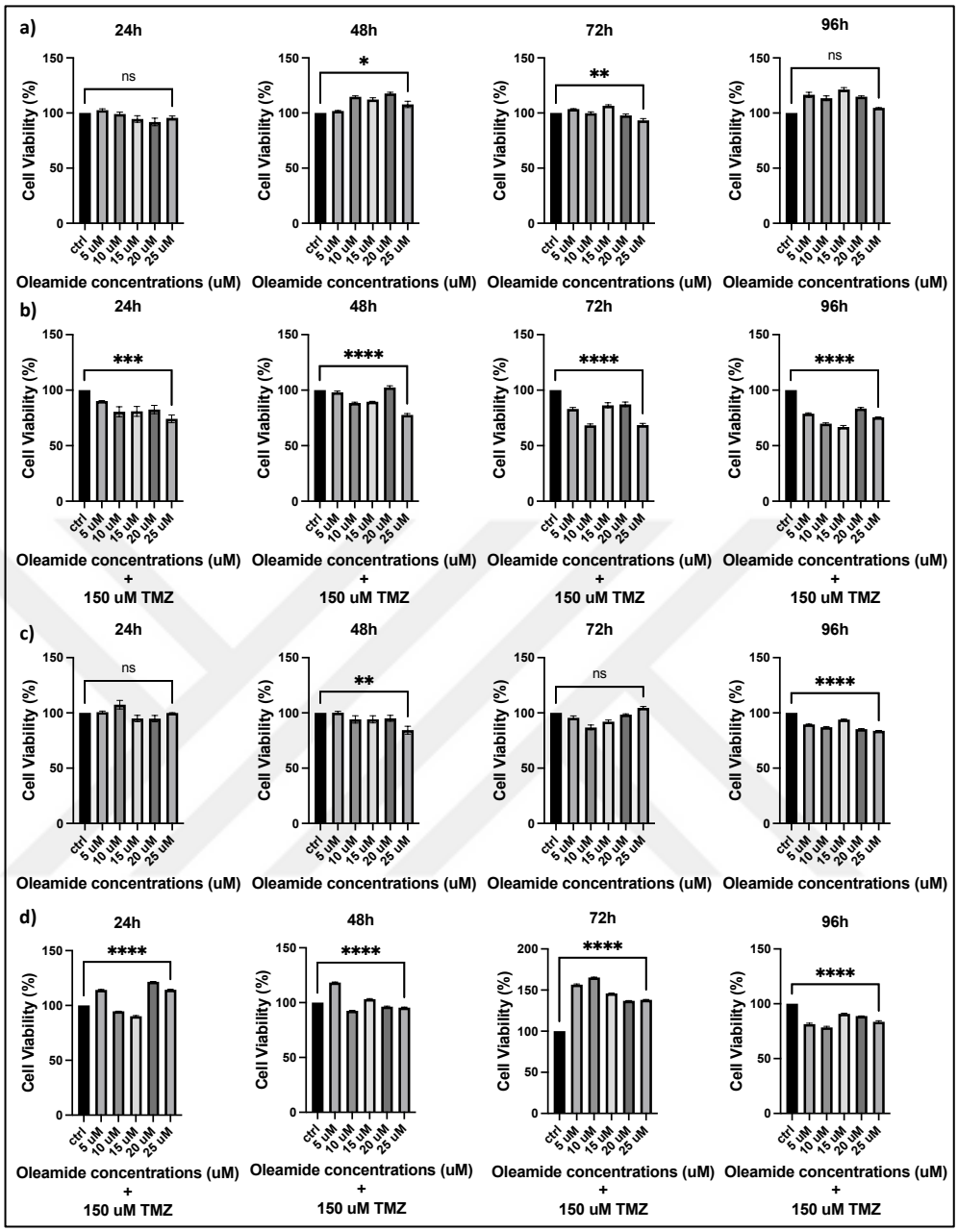

Figure 3.4. Cell viability of GBM cell lines a and b represent A172, while c and d represent U87 cell line. a) A172 cells which were treated with oleamide with the concentration range of 5 uM to 25 uM b) oleamide + 150 uM of TMZ for 24h, 48h, 72h, 96h incubation and c) U87 cells which were treated with oleamide d) oleamide + 150 uM of TMZ.

As shown in Figure 3.4., combination of oleamide with TMZ had better effect on GBM cell lines than using only TMZ and oleamide. Interestingly, viability of astrocytes increases in the presence of TMZ compared to using only oleamide, shown in Figure 3.3.. According to

data, 25 uM concentration of oleamide might have an effect on GBM cells compared to other oleamide concentrations.

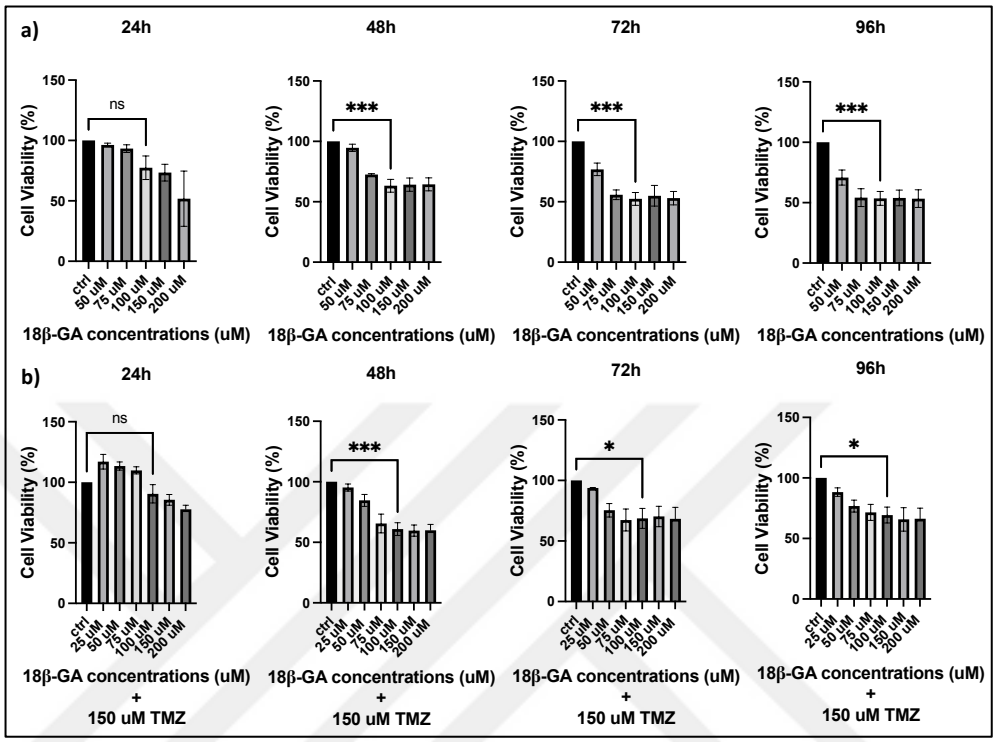

Figure 3.5. Cell viability of Immortalized Human Astrocytes cells which were treated with a) 18β-GA with the concentration range of 25 uM to 200 uM and b) 18β-GA + 150 uM of TMZ for 24h, 48h, 72h, 96h incubation.

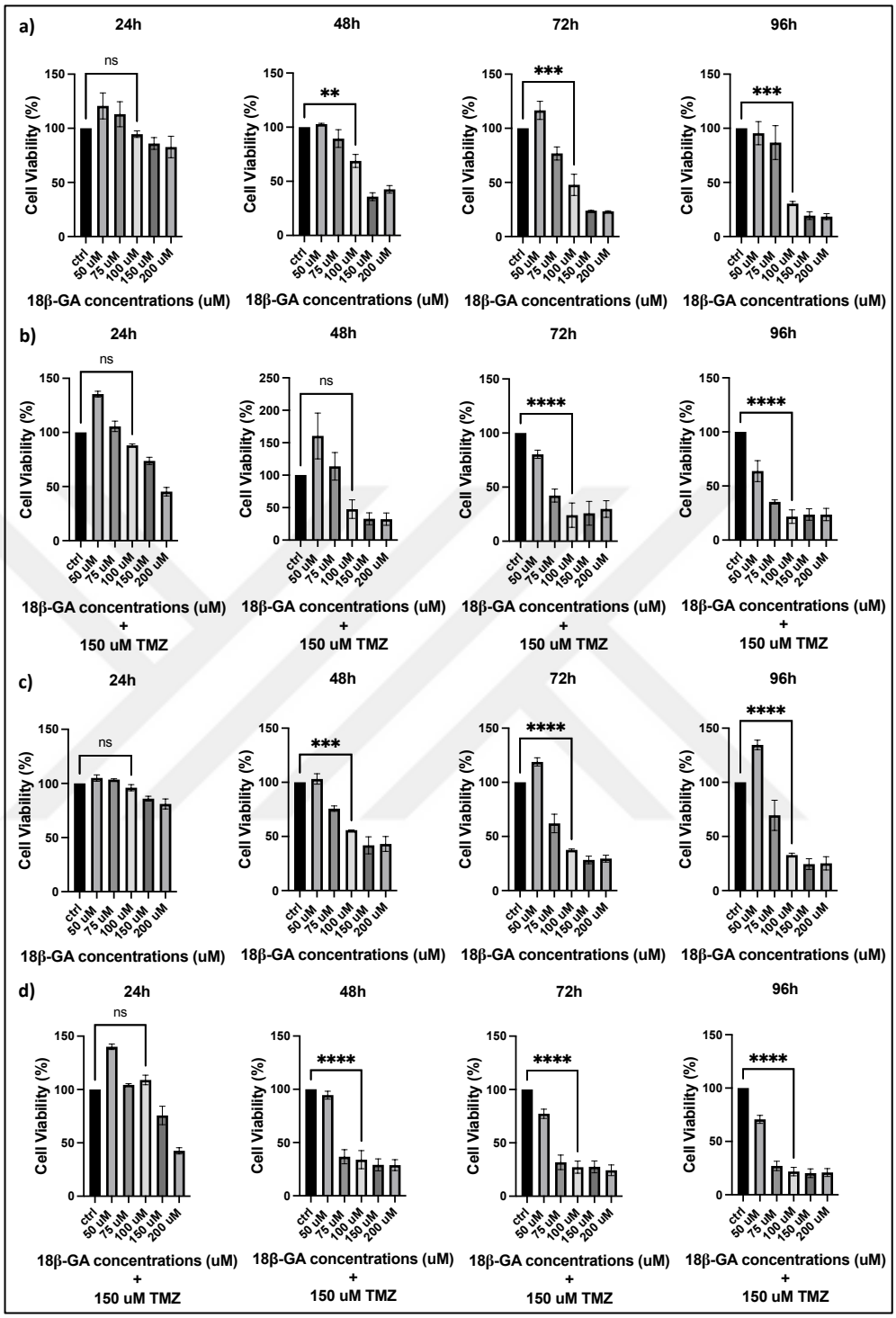

Figure 3.6. Cell viability of GBM cell lines, a and b represent A172, while c and d represent U87 cell line. a) A172 cells which were treated with 18β-GA with the concentration range of 50 uM to 200 uM b) 18β-GA + 150 uM of TMZ for 24h, 48h, 72h, 96h incubation and c) U87 cells which were treated with 18β-GA d) 18β-GA + 150 uM of TMZ

As shown in Figure 3.6., combination of 18β-GA with TMZ had better effect on GBM cell lines than using only TMZ and 18β-GA. Interestingly, viability of astrocytes increases in the presence of TMZ compared to using only 18β-GA, shown in Figure 3.5.. According to data, 100 uM concentration of 18β-GA might have an effect on GBM cells compared to other oleamide concentrations.

Overall results showed that, appropriate concentration of oleamide is 25 uM, while the effective concentration of 18β-GA is 100 uM in the presence of TMZ with 150 uM concentration. The concentrations were determined based on the viability of healthy cells and dose that GBM cells were more damaged.

3.2. CO-CULTURE EXPERIMENTS BY FLOW CYTOMETRY

Determining the astrocytes and GBM interaction, co-culture experiments were done with and without the gap junction inhibitor, 25 uM concentration of oleamide, in order to determine its effect on cell to cell communication. Figure 3.7. indicates the flow cytometry results.

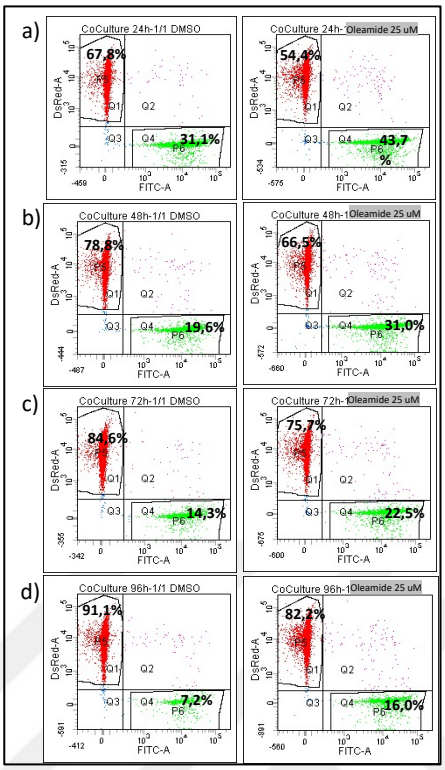

Figure 3.7. Flow cytometry analysis of cell viability of co-cultured eGFP astrocytes and dsRed A172 cells in 1:1 ratio with DMSO control and the presence of oleamide at a) 24h b) 48h c)72h d) 96h incubation.

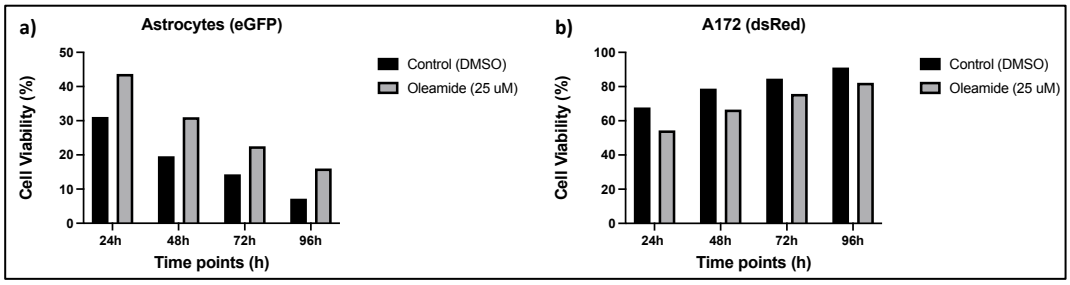

Figure 3.8. Cell viability of co-cultured a) eGFP astrocytes and b) dsRed A172 cells in 1:1 ratio with DMSO control and the presence of oleamide at 24h, 48h, 72h, 96h incubation.

As seen in Figure 3.7. and Figure 3.8., gap junction inhibitor increases the cell viability of astrocytes and decreases the GBM cell viability by affecting the cell to cell communication.

3.3. WESTERN BLOT ANALYSIS

The membrane images of the samples and their bar graphs are shown below. All of the samples are numbered from 1 to 6 in the same order; control group, oleamide group, oleamide+TMZ group, TMZ group, 18β-GA group and 18-b-GA+TMZ group, respectively.

3.3.1. Cx43 protein levels

According to data, protein level of Cx43 mostly decreases in astrocytes when combined treatment with 18β-GA and TMZ was applied, as shown in Figure 3.9., significant change was not be observed in TMZ group.

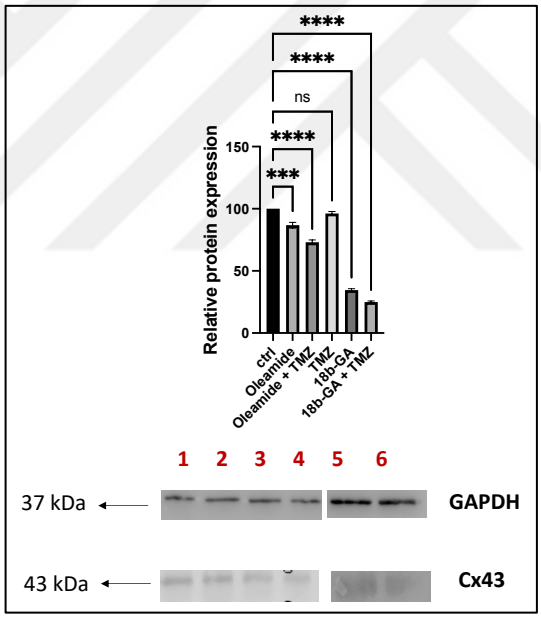

Figure 3.9. Western blot images of Cx43 protein levels in Immortalized Human Astrocytes cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively.

According to data represented in Figure 3.10., protein level of Cx43 in A172 cell line mostly increases in TMZ group compared to combined treatment with TMZ+18β-GA and TMZ+oleamide. Protein level of Cx43 in U87 cell line mostly increases in combined

treatment with TMZ+18β-GA. In TMZ group, data found in the same figure, significant change was not be observed for protein level of Cx43 compared to control group.

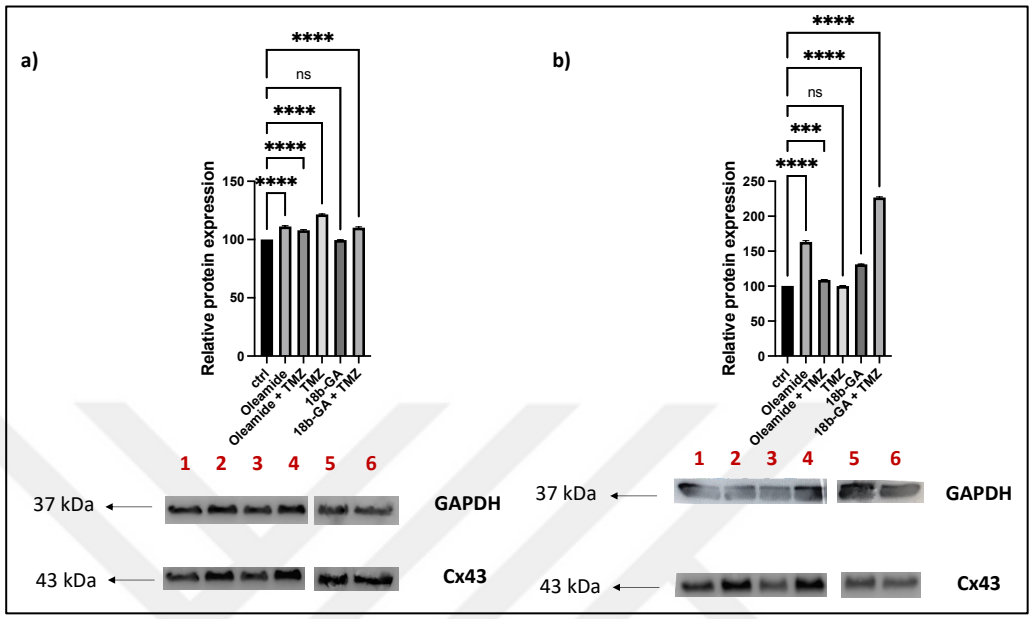

Figure 3.10. Western blot images of Cx43 protein levels in a) A172 cells and b) U87 cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively.

3.3.2. Caspase-3 and Cleaved Caspase-3 Protein Levels

According to data, protein level of Cas3 decreases in astrocytes when combined treatment with oleamide and TMZ was applied, as shown in Figure 3.11.. Protein level of Cas3 decreases also in TMZ group compared to control group, while is slightly decreased in 18β-GA +TMZ group. However, Cas3 protein level is mostly decreased in 18β-GA group in astrocytes. According to data, protein level of Cleaved Cas3 decreases in astrocytes while combined treatments were applied, as shown in Figure 3.11.. Cleaved Cas3 protein level is mostly decreased in 18β-GA group. However, protein level of Cleaved Cas3 significantly increases in TMZ group.

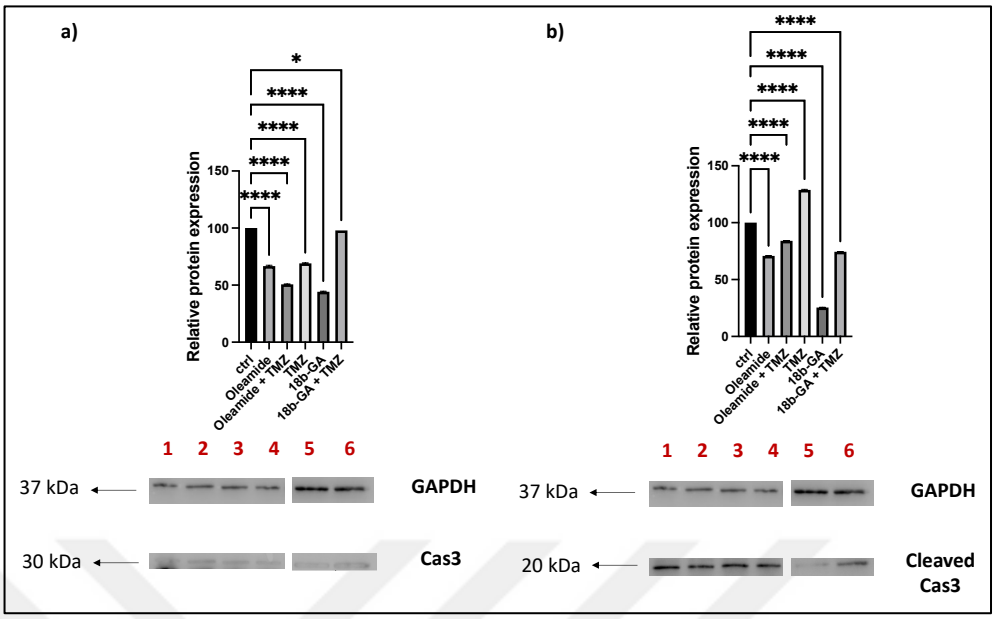

Figure 3.11. Western blot images of a) Cas3 protein levels and b) Cleaved Cas3 protein levels in Immortalized Human Astrocytes cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively.

According to data, protein level of Cas3 in A172 cell line decreases only in 18β-GA group, shown in Figure 3.12.. It increases in other groups, while there is no significant change in 18β-GA+TMZ group. According to data, protein level of Cleaved Cas3 in A172 cell line decreases all groups, shown in Figure 3.12.. However, mostly decreases in TMZ group. According to data, protein level of Cas3 decreases in U87 cell line in TMZ group, while Cas3 protein level mostly increases in combined treatment of 18β-GA+TMZ group, as shown in Figure 3.12.. In oleamide group, there is also a rise in Cas3 protein. According to data, protein level of Cleaved Cas3 in U87 cell line increaes all groups except TMZ group, shown in Figure 3.12. However, maximum rise is observed in combined treatment groups.

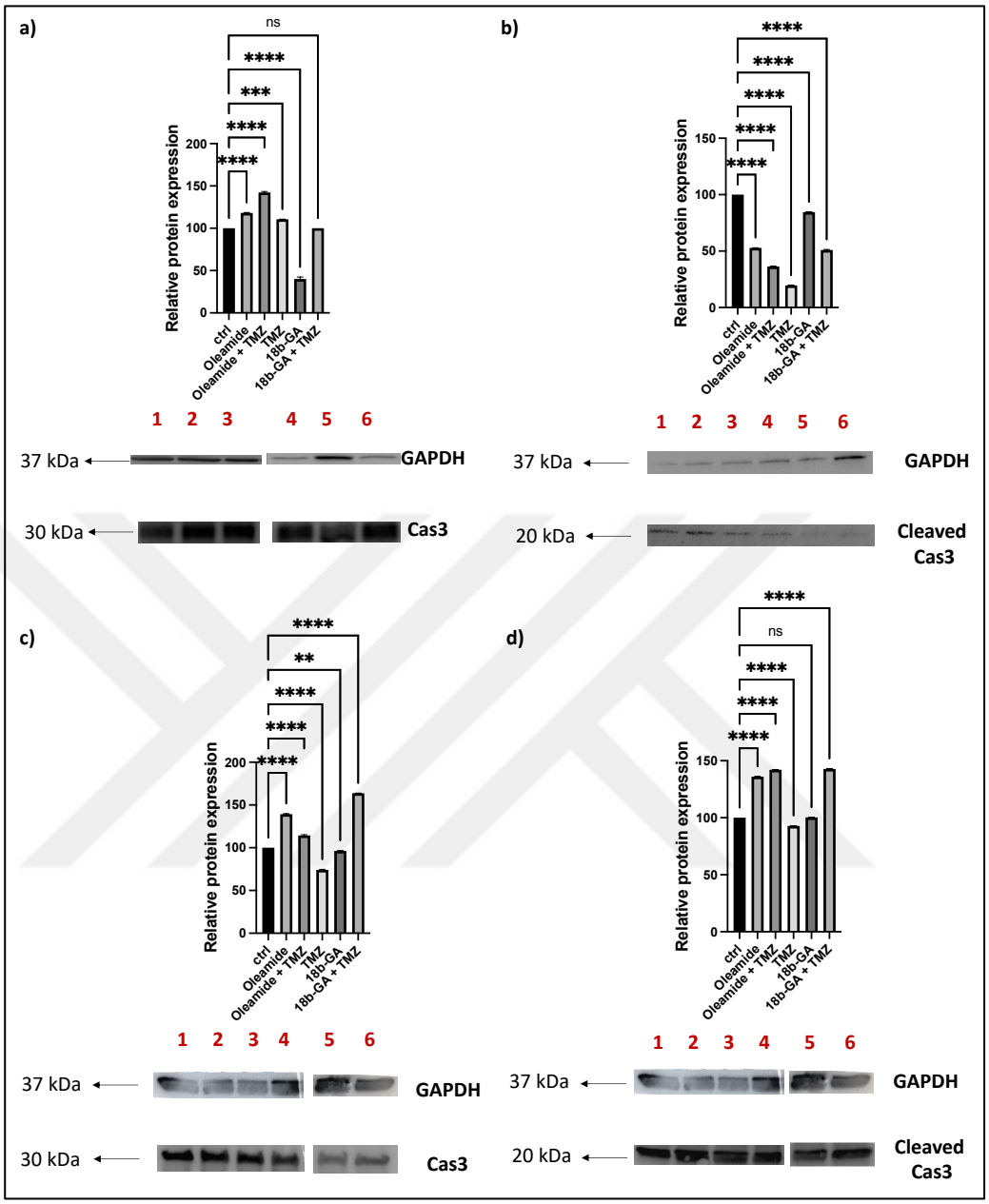

Figure 3.12. Western blot images of Cas3 protein levels in a) A172 cells and c) U87 cells, and Cleaved Cas3 protein levels in b) A172 cells and d) U87 cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively.

3.3.3. Caspase-7 and Cleaved Caspase-7 Protein Levels

According to data, protein level of Cas7 decreases in astrocytes when combined treatment of 18β-GA+TMZ was applied, as shown in Figure 3.13.. However, in other groups, Cas7 protein level increases, especially in combined treatment of oleamide+TMZ and TMZ groups. According to data, protein level of Cleaved Cas7 decreases in astrocytes mostly in 18β-GA group, as shown in Figure 3.13.. It is observed that Cleaved Cas7 protein level only increases in TMZ group.

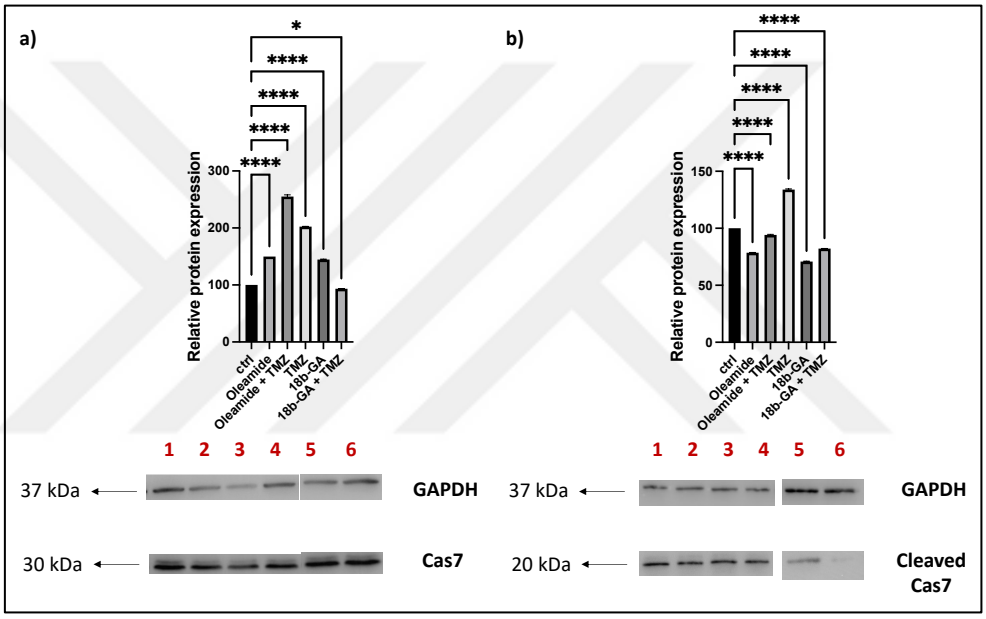

Figure 3.13. Western blot images of a) Cas7 protein levels and b) Cleaved Cas7 protein levels in Immortalized Human Astrocytes cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively.

According to data, protein level of Cas7 increases in A172 cell line when treated with 18-b-GA, as shown in Figure 3.14., while it mostly decreases in TMZ group. In other groups, Cas7 protein levels decrease, while in combined treatment with oleamide+TMZ, slightly decreases. According to data, protein level of Cleaved Cas7 increases in A172 cell line when oleamide, 18β-GA and combined treatment of 18β-GA +TMZ were applied, as shown in Figure 3.14., while it mostly increases in TMZ group. In oleamide+TMZ groups, Cleaved Cas7 protein levels decreases. According to data, protein level of Cas7 nearly two fold

increases in U87 cell line when combined treatment of 18β-GA +TMZ was applied, as shown in Figure 3.14., while it mostly decreases in TMZ group. In oleamide and 18β-GA groups, Cas7 protein levels increase, while in combined treatment with oleamide+TMZ, it slightly decreases. According to data, protein level of Cleaved Cas7 mostly increases in U87 cell line when combined treatment of 18β-GA +TMZ was applied, as shown in Figure 3.14., while it mostly decreases in TMZ group. In oleamide and oleamide+TMZ groups, Cleaved Cas7 protein levels increase, while in 18β-GA group, it slightly decreases.

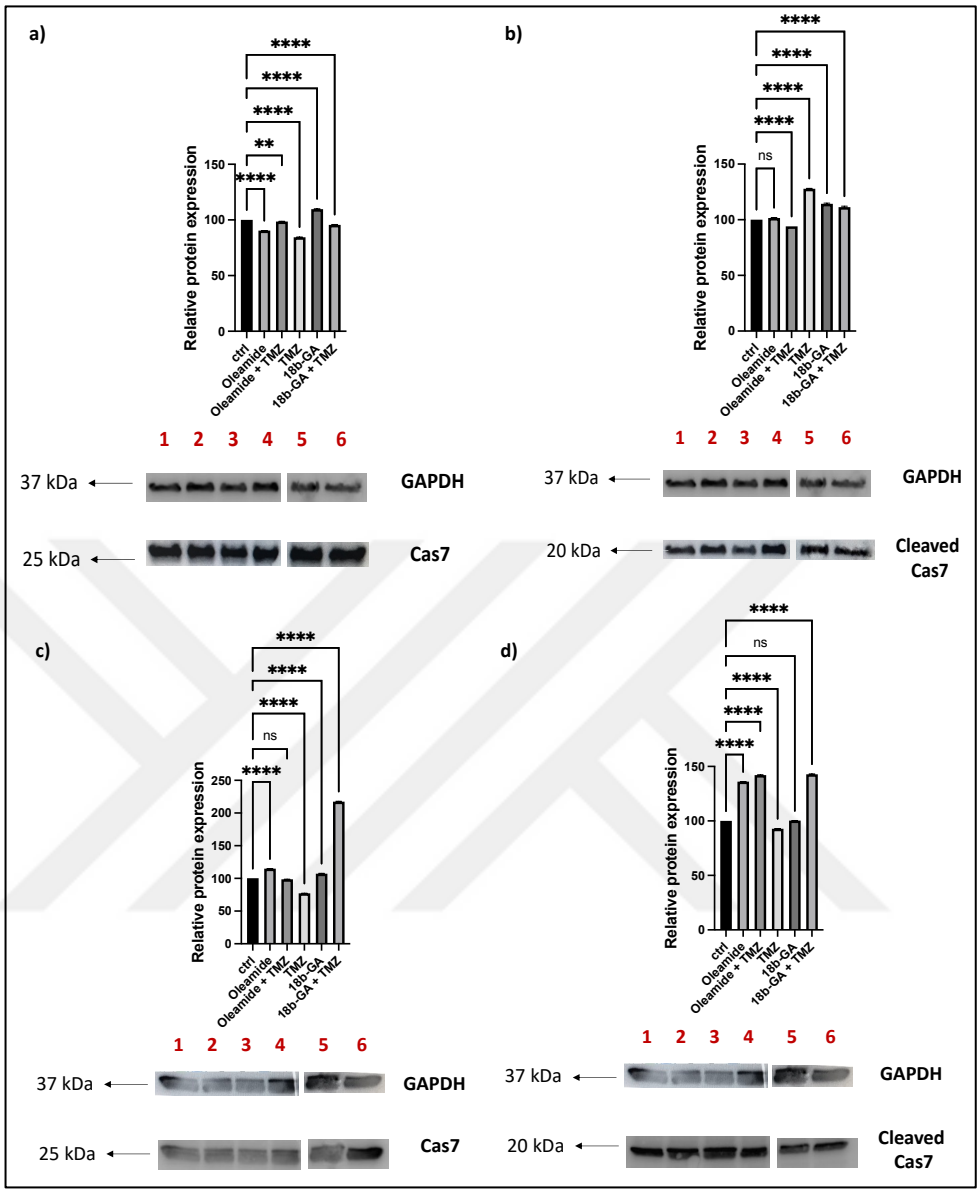

Figure 3.14. Western blot images of Cas7 protein levels in a) A172 cells and c) U87 cells, and Cleaved Cas7 protein levels in b) A172 cells and d) U87 cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively.

3.3.4. JAK1, STAT3 and pSTAT3 Protein Levels

According to data, protein level of JAK1 in astrocytes increases in all groups, mostly in 18β-GA +TMZ group, as shown in Figure 3.15.. However, JAK1 protein increases slightly in

TMZ group compared to other groups and control group. According to data, protein level of STAT3 in astrocytes increases in 18β-GA and especially in 18β-GA+TMZ group, as shown in Figure 3.15.. However, data could not be handled for other groups. According to data, protein level of pSTAT3 in astrocytes increases in oleamide group and 18β-GA group, but mostly increases in 18β-GA +TMZ group, as shown in Figure 3.15.. pSTAT3 protein level decreases in oleamide+TMZ gorup and mostly decreases in TMZ group.

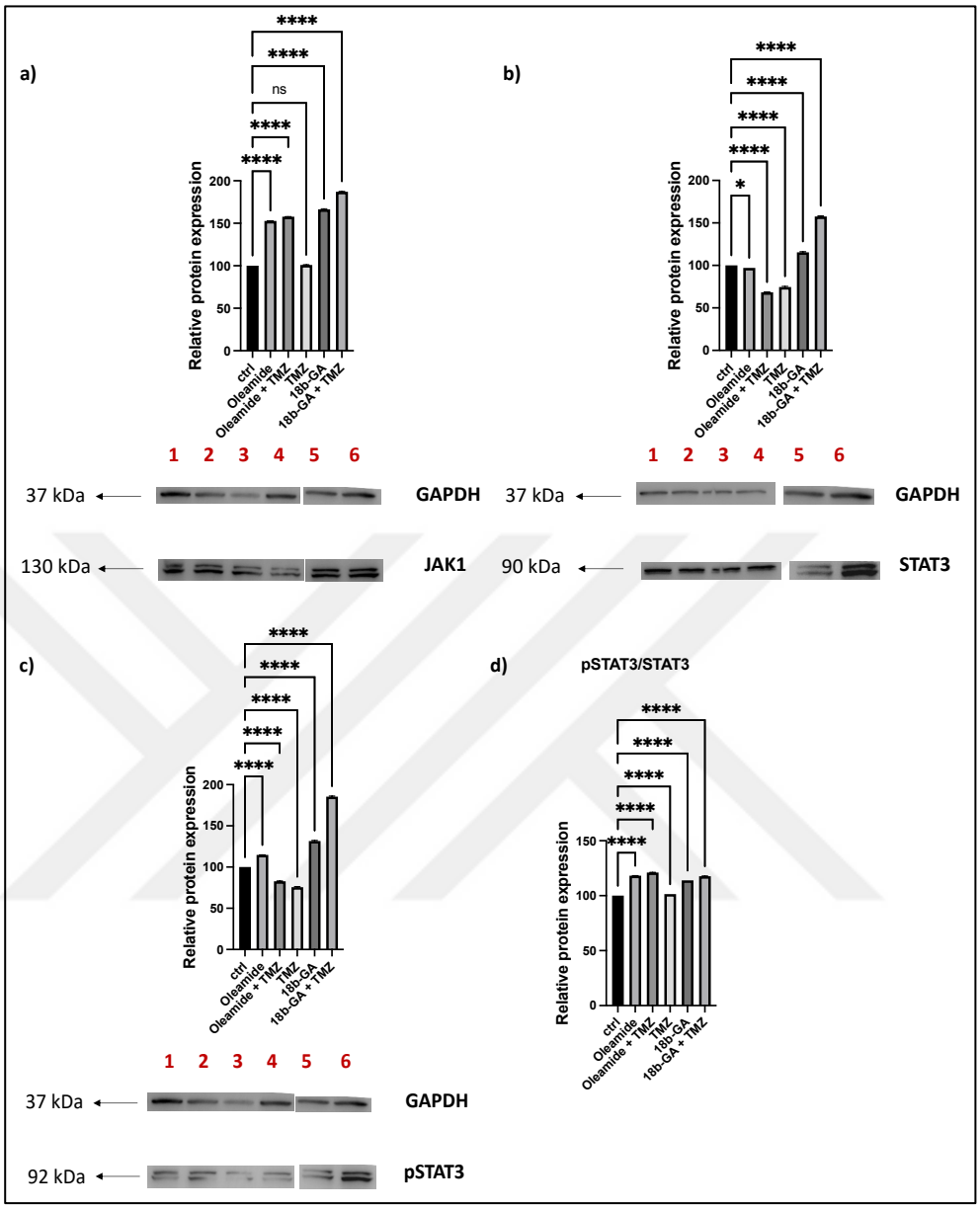

Figure 3.15. Western blot images of a) JAK1 protein levels, b) STAT3 protein levels and c) pSTAT3 protein levels in Immortalized Human Astrocytes cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively. d) Relative protein expression of pSTAT3 to STAT3

According to data, protein level of JAK1 in A172 cells mostly decreases in TMZ group. JAK1 also decreases in oleamide and oleamide+TMZ group, as shown in Figure 3.16.. However, JAK1 protein level slightly decreases in 18β-GA and 18β-GA +TMZ group. According to data, protein level of STAT3 in A172 increases in 18β-GA and 18β-GA +TMZ

groups, while it decreases in other groups, especially in oleamide+TMZ group, as shown in Figure 3.16 . According to data, protein level of pSTAT3 in A172 increases in TMZ and 18β-GA groups, while it decreases in oleamide and oleamide+TMZ group, as shown in Figure 3.16. Protein level of pSTAT3 mostly increases in 18β-GA+TMZ group. Relative protein expression of pSTAT3/STAT3 also increased in A172 cell line.

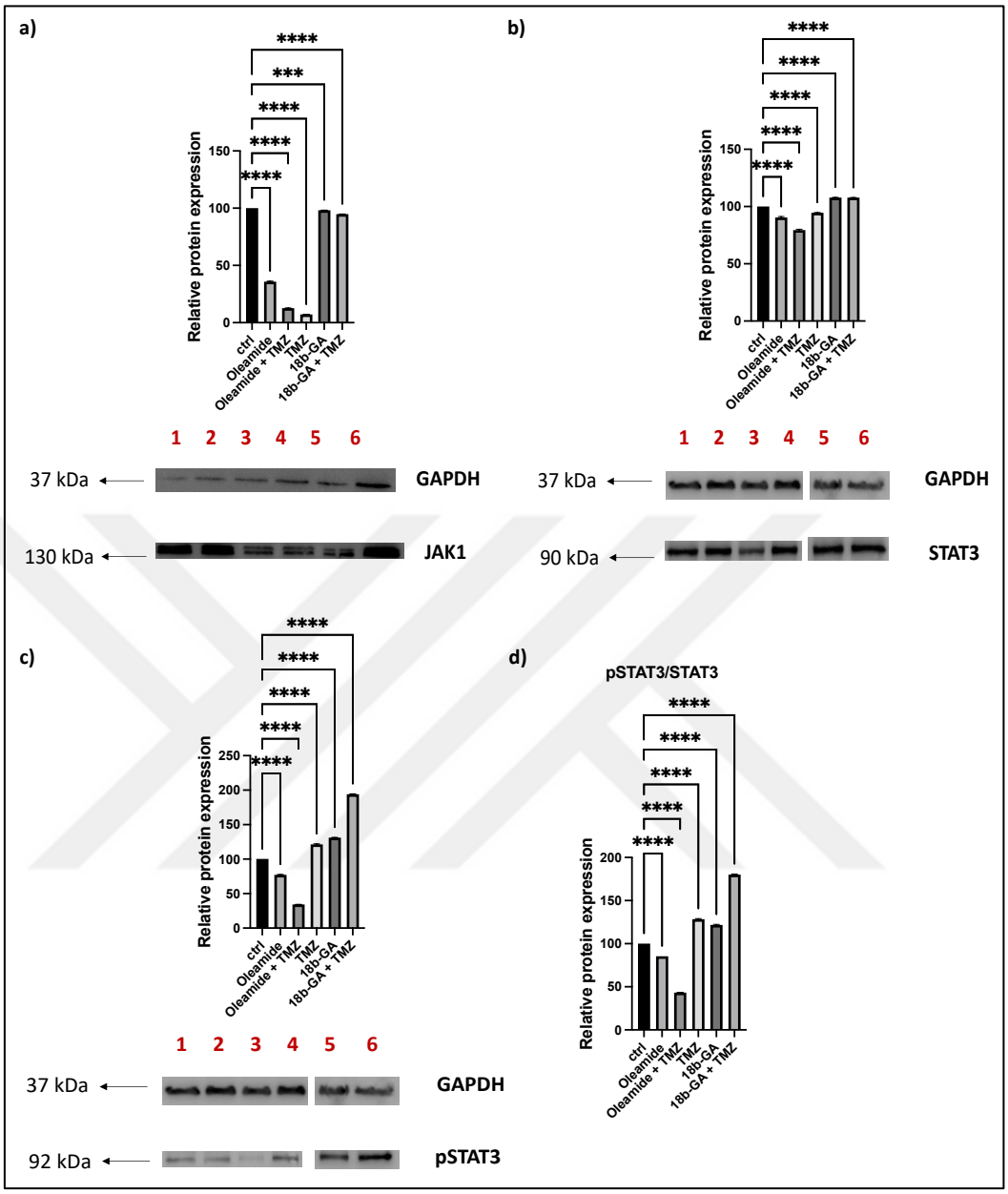

Figure 3.16. Western blot images of a) JAK1 protein levels, b) STAT3 protein levels and c) pSTAT3 protein levels in A172 cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively. d) Relative protein expression of pSTAT3 to STAT3.

According to data, protein level of JAK1 in U87 increases in all groups, except 18β-GA +TMZ group, as shown in Figure 3.17.. It mostly increases in oleamide treated group. According to data, protein level of STAT3 in U87 increases in all groups except TMZ group,

and the protein level increase mostly in 18β-GA +TMZ group, as shown in Figure 3.17.. According to data, protein level of pSTAT3 in U87 increases in all groups, except TMZ group, but mostly increases in 18β-GA +TMZ group, as shown in Figure 3.17.. However, relative protein expression of pSTAT3/STAT3 decreased in U87 cell line.

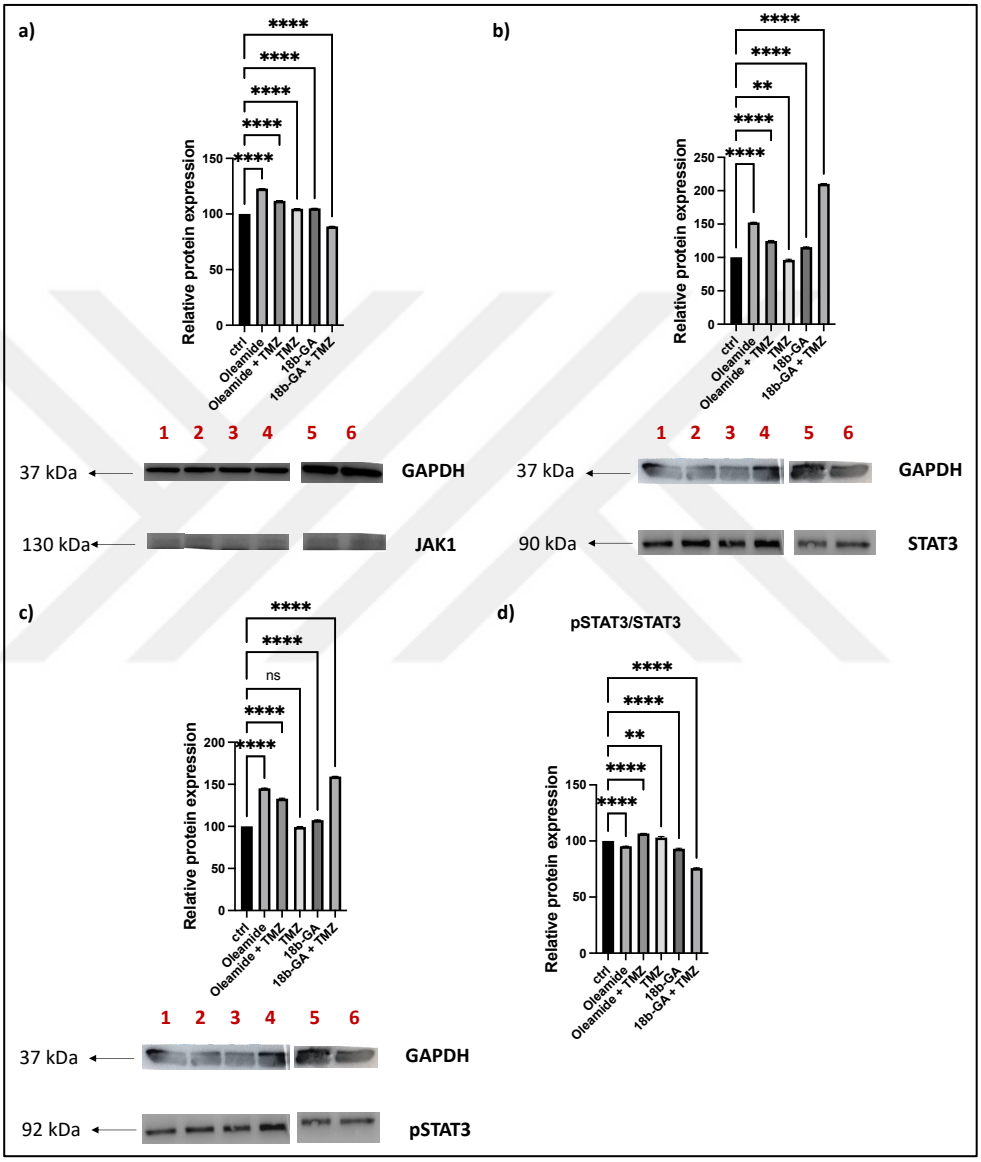

Figure 3.17. Western blot images of a) JAK1 protein levels, b) STAT3 protein levels and c) pSTAT3 protein levels in U87 cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively. d) Relative protein expression of pSTAT3 to STAT3.

3.3.5. Wnt5a Protein Levels

According to data, protein level of Wnt5a in astrocytes increases in all groups, but especially in oleamide+TMZ group, as shown in Figure 3.18..

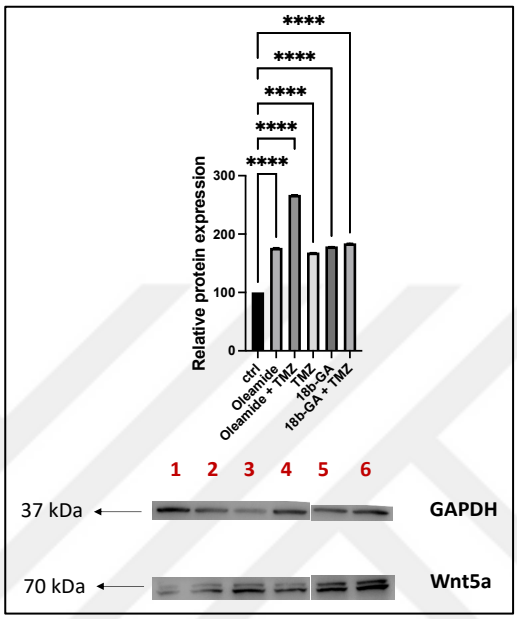

Figure 3.18. Western blot images of Wnt5a protein levels in Immortalized Human Astrocytes cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively.

According to data, protein level of Wnt5a in A172 cell line increases in 18β-GA group, but especially in 18β-GA+TMZ group, as shown in Figure 3.19., while it decreases in oleamide and TMZ groups. Protein level of Wnt5a decreases mostly in oleamide+TMZ group. According to data, protein level of Wnt5a in U87 cell line increases in all groups, but especially in 18β-GA+TMZ group, as shown in Figure 3.19..

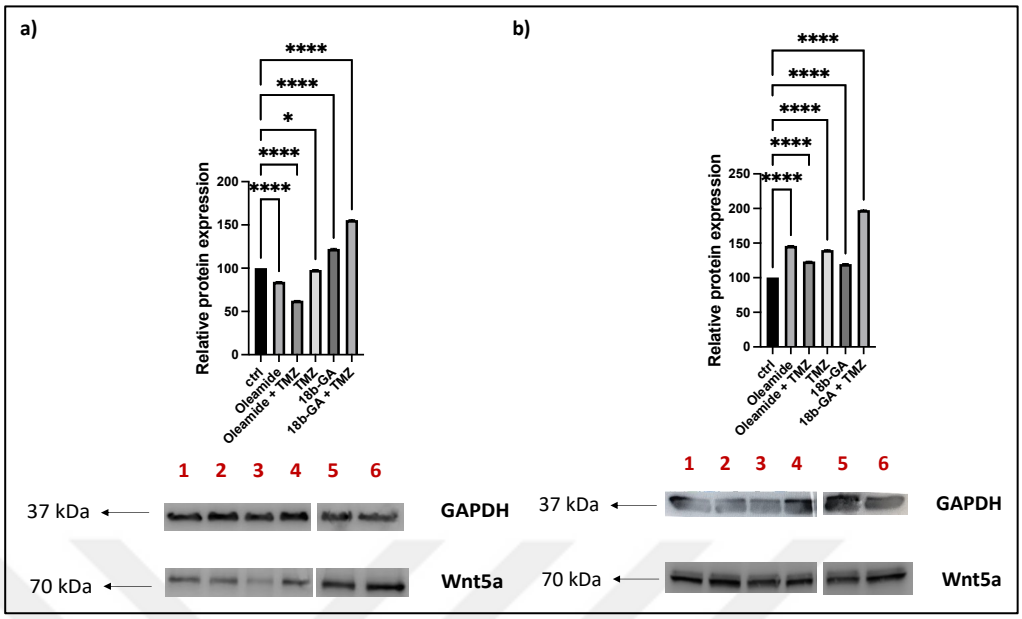

Figure 3.19. Western blot images of Wnt5a protein levels in a) A172 cells and b) U87 cells after treatment of oleamide, TMZ and 18β-GA. 1) Control group 2) oleamide (25 uM), 3) oleamide (25 uM) + TMZ (150 uM), 4) 150 uM TMZ, 5) 100 uM 18β-GA, 6) 18β-GA (100 uM) + TMZ (150 uM), respectively.

4. DISCUSSION

The most aggressive form of gliomas, that derived from glia or their precursors in the central nervous system, is glioblastoma (GBM) [114]. For tumor progression, glioblastoma disturb normal functions of healthy brain cells by recruiting them and generate a microenvironment. In this microenvironment, cancerous cells contact with normal cells, other cancerous cells and immune cells in order to encourage proliferation of tumor, metastasis, resistance to therapy, angiogenesis and suppression of immune cells. In direct communication through such as cytokines and direct communication between cells generate this contact [115]. Gap junctions are responsible for direct interaction between cells and Connexin-43 is the mostly found gap junction protein in glioblastoma [116]. Gap junction inhibitors interrupt the communication between cells and prevent transferring of substances from one cell to each other. Oleamide and 18β-Glycyrrhetinic acid are the examples of these gap junction blockers and both of them block Connexin-43 [117,118]. Temozolomide (TMZ), which is the only chemotherapeutical used in glioblastoma and, newly emerged studies indicate that combination of TMZ with other molecules is more effective than using it alone [119,120]. The effects of gap junction inhibitors and their interaction with TMZ were examined since gap junctions play a significant role in GBM. The gap junction inhibitor, oleamide, decreased the viability of astrocytes fewer, compared to control group, by affecting the direct communication between two cells. According to protein, Cx43 protein level decreases in astrocytes, while increases in GBM cell lines. While increasing protein level of Cx43 is associated with disease progression [67], this increase is related with chemoresistance in GBM [69]. Considering these results, it was observed that the combined treatment reduced the disease progression, while it lead to countercheck to treatment. Furthermore, oleamide and 18β-GA lead to increase Cx43 protein level and cause blood brain barrier (BBB) disruption [121,122]. Cx43 proteins found in astrocytes provide integrity of BBB. Therefore, Cx43 protein level decrease in astrocytes lead to increase BBB permeability [123]. Recent drug developments that target brain tumors are conducting based on BBB disruption in order to increase the efficiency of drugs. Several drugs such as mannitol and RMP-7 are designed to disrupt BBB for an effective treatment [124]. In the light of this information, gap junction inhibitors, oleamide and 18β-GA, might be used as helper substances in order to increase drug efficiency. Apoptosis markers were completely reduced in astrocytes in combined treatment group, it increases in GBM cells, accordingly causes cell death. One of the studies

demonstrated that oleamide increases apoptosis, after increasing Cx43 protein level [121,125]. Due to the significance of signaling pathways in progression of GBM, JAK1/STAT3, phosphorylated form of STAT3 (pSTAT3) and Wnt5a protein analyzes were performed to reveal how combined treatments affect these signaling pathways. JAK/STAT related proteins increased in astrocytes, in combined treatment. JAK/STAT pathway is related with immunological response, accordingly, under the stressing conditions, this pathway is activated in astrocytes [126]. STAT3 and its phosphorylated form also increased in GBM, and is highly associated with chemoresistance [93]. However, relative protein expression of pSTAT3 to STAT3, that indicates activity of JAK/STAT pathway, decreases in U87 cell line while it increases in A172 cell line. That difference between two GBM cell line might be caused by their subtypes. A172 cell line is proneural subtype, while U87 cell line is mesenchymal subtype [31,127,128]. Wnt5a protein level increased both in astrocytes and GBM in combined treatment groups. Normally, Wnt5a protein level is elevated in GBM and plays a crucial role in chemoresistance by encouraging self-renewal of glioma stem cells (GSCs) and EMT process [91]. In one study, it is represented that, Wnt5a protein level is positively associated with cancer-associated inflammation in astrocytes [129]. In this thesis, functional experiments regarding to inflammation response did not be studied. However, cancer-associated inflammation might exacerbate progression of GBM. Even cell viability was significantly decreased in combined therapy, the results of protein expressions indicated that long time treatments for evaluation of cell viability. In this study, it has been demonstrated that gap junction inhibitors which interrupt the direct communication between two cells combined with TMZ lead to apoptosis of GBM cells rather than healthy cells. However, remaining cells may develop a resistance to treatment.

5. CONCLUSION

In conclusion, when gap junction inhibitors are combined with TMZ lead to reduced cell viability in GBM cells rather than astrocytes cells. Interestingly, despite the reduced viability of GBM cells in combined treatment, cells also exhibit a protein expression profile in which chemoresistance related proteins are elevated. The results indicate the need of expanded time studies for combined treatment since the remaining cells might have increased chemoresistance. Apparently, TMZ and Cx43 inhibitors used in this study has synergist effects on GBM cell viability in short term but the chemical interactions and long time periods must be included in order to assess their clinical availability.

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