T.C.
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M.Sc. THESIS

THE EFFECTS of TEMOZOLOMIDE on EXOSOMAL STRESS PROTEINS in GLIOMA CELLS

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FOREWORD

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Explanation
%	: Percent
~	: Approximately
<	: Less than
>	: Greater than
µL	: Microliter
µM	: Micromolar
cm^2	: Square centimeter
kDa	: Kilodalton
kg	: Kilogram
L	: Liter
M	: Molar
mg	: Milligram
mL	: Milliliter
mM	: Millimolar
ng	: Nanogram
nm	: Nanometer
nM	: Nanomolar
ºC	: Degree Celsius
V	: Volt
v	: Volume
w	: Weight

Abbreviation	Explanation
AcOH	: Acetic acid
APS	: Ammonium persulfate
BBB	: Blood-brain barrier
BCA	: Bicinchoninic acid
BER	: Base excision repair
BSA	: Bovine serum albumin
CNS	: Central nervous system
CO_2	: Carbondioxide
D-PBS :	: Dulbecco's phosphate buffered saline
ddH_2O	: Double distilled water
dH_2O	: Distilled water
DMEM	: Dulbecco's modified eagle's medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
EDTA	: Ethylenediaminetetraacetic acid
EGFR	: Epidermal growth factor receptor
EV	: Extracellular vesicles
FBS	: Fetal bovine serum
FDA	: Food and drug administration (US)
GAPDH	: Glyceraldehyde 3-phosphate dehydrogenase
GBM	: Glioblastoma multiforme
HGNC	: Human gene nomenclature committee
HRP	: Horseradish peroxidase
HSF	: Heat shock factor
Hsp	: Heat shock protein
IC_{50}	: Inhibitory concentration (half maximal)
LAF	: Laminar air flow
MeOH	: Methanole
MGMT	: O_6 Methylguanine DNA methyltransferase
min	: Minute

miRNA : MicroRNA

mRNA : Messenger RNA

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

MVB : Multivesicular body

NaCl : Sodium chloride

NEAA : Non-essential amino acids

NF-κB : Nuclear factor kappa B

NP-40 : Nonidet P40

PVDF : Polyvinylidene difluoride membrane

RNA : Ribonucleic acid

Rpm : Revolutions per minute

SDS : Sodium dodecyl sulfate

SDS-PAGE : Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

TMZ : Temozolomide

UV : Ultraviolet

WHO : World health organization

ÖZET

YÜKSEK LİSANS TEZİ

TEMOZOLOMİDİN GLİOMA HÜCRELERİNDEKİ EKSOZOMAL STRES PROTEİNLERİ ÜZERİNE ETKİLERİ

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Beyin tümörleri, tüm dünyada ölümcül sonuçları olan kanser türlerinden biridir. Gliomalar, merkezi sinir sisteminin glial hücrelerinden meydana gelen ve en sık görülen beyin tümörleridir, ve en agresif yaygın primer beyin malignitesi, glioblastoma multiform (GBM, derece IV astrositom) olarak adlandırılmıştır. GBM hastaları için ortalama hayatta kalma süreleri bir yıldan az olarak öngörülmektedir. Ameliyata, radyoterapötik ve kemoterapötik müdahalelere rağmen, sağ kalım oranları dikkate değer bir değişiklik göstermemektedir. Bu nedenle, dünya çapında, bilim adamları ve klinisyenler malign gliomalar için hala daha iyi tedaviler aramaktadır. Kan-beyin bariyerinin (KBB) varlığı kemoterapinin etkinliğini azaltmada önemli bir rol oynamaktadır. Bir DNA alkilleyici ajan olan Temozolomid (TMZ) yapısı itibariyle KBB'ye nüfuz edebildiğinden dolayı, tekrarlayan glioma için en umut verici tedavi olarak kullanılan kemoterapötiktir.

Son zamanlarda yapılan kanser araştırmalarında, beyin tümörlerinin tedavileri Hsp seviyelerini düşürmeye yönelik olarak şekillendirilmeye başlanmıştır. Bunun nedenleri arasında, beyin tümörleri ile stres proteinleri arasında bir ilişki olduğunun ortaya konulması, bu proteinlerin tümör hücrelerinin çoğalmasında ve progresyonunda etkili olması ve bu proteinlerin miktarlarındaki artışa bağlı olarak kanser hücrelerinin giderek tedaviye karşı direnç göstermesi sayılabilir. Bu proteinlerin anlatımının baskılanmasının tedaviye sağlayacağı katkı nedeniyle stres proteinleri yeni ilaçların geliştirilmesinde önemli bir odak noktası haline gelmiştir. Literatürde farklı yöntemler kullanılarak bu proteinlerin anlatımlarının baskılanmasını sağlayan birçok çalışma bulunmaktadır. Bu yöntemler arasında TMZ ajanı ile ısı şoku proteinlerinin

anlatım seviyesini düşürmeyi hedeflemek de yer almaktadır. Yeni yapılan araştırmalar hücrelerin, hücre dışı veziküller (EV'ler) vasıtasıyla da iletişim kurabildiğini göstermektedir. 30-150 nm çapında ve lipit çift katmanıyla çevrili küresel yapılara sahip olan eksozomlar EV ailesinin en çok araştırılan grubu haline gelmiştir. Eksozomların kanser progresyonda önemli rolleri olduğu ve tümör mikro ortamını içerdikleri (DNA, RNA ve proteinler- bazı Hspler dahil) çeşitli materyaller ile değiştirebildikleri yapılan çalışmlarda gösterilmiştir. Bu nedenle, kansere bağlı eksozomların ve eksozom yüzeyi HSP'lerinin terapötik bir hedef olabileceği düşünülmektedir.

Bu çalışmanın temel amacı, TMZ'nin, U87MG hücrelerinden salınan eksozomlarda HSP'lerin ekspresyonu üzerindeki etkilerini değerlendirmektir. Çalışmanın başında TMZ'nin hücre canlılığı üzerindeki etkisi incelendi. Sitotoksisite analizlerine göre, ajan, hücreler üzerinde toksik veya proliferatif bir etki göstermeyen (%100 hücre canlılığı), hücre canlılığını %20'den az oranda etkileyen ve %50 oranında etkileyen konsantrasyonlarda hücrelere uygulandı. Eksozom izolasyonu yapıldıktan sonra, TMZ uygulamasının hem eksozomal hem de hücre kaynaklı çözünür protein profilleri üzerindeki etkisi 1 boyutlu elektroforez tekniği ile gösterildi. Proteinler bu jeller üzerinde ayrıldıktan sonra Western blot tekniği ile membrana aktarıldı ve hedef proteinler için spesifik antikorlarla inkübe edildi. İzolasyonun verimi, Western blot tekniği kullanılarak en yaygın eksozomal marker proteinleri ile ve SEM görüntüleri ile gösterildi. Eksozomların metaztazdaki rolünü açıklamak için hücre migrasyonu deneyi yapıldı. Sonuçların, en yüksek dozda TMZ uygulamasıyla hücre göçünün, dolayısıyla ekstraselüler Hsp70 miktarını düşmesine bağlı olarak anjiyogenezin azalmasını yansıtacak umut vaddedici bir yaklaşım olduğu öngörülmektedir. Bu çalışmanın sonuçlarına göre, glioma hücrelerine uygulanan çeşitli konsantrasyonlardaki TMZ'nin doza bağlı olarak hücre içi Hsp70'in anlatım seviyesini artırdığı görülürken, hücre dışı-eksozomal yolakla salgılanan Hsp70'in anlatım seviyesini azalttığı görülmüştür.

U87MG glioma hücrelerinde Hsp70 anlatımını en çok artıran ve eksozomal Hsp70'in anlatımın en çok azaldığı TMZ konsantrasyonun 750 μM olduğu belirlenmiştir. Bu sonuçlar, hücrelere uygulanan kemoterapi ajanın beyin tümörleri tedavileri için etkin bir terapötik seçenek olabileceğini göstermektedir. Bu çalışmadan elde edilen veriler yüksek ölüm oranı gözlenen beyin tümörlerinin tedavisi için yenilikçi tedavilerin geliştirilmesine katkı sağlayacaktır.

Temmuz 2019, 74 sayfa.

Anahtar kelimeler: Temozolomid (TMZ), glioma, eksozom, Hsp70, hücre migrasyonu.

SUMMARY

M.Sc. THESIS

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Brain tumors are one of the cancer types with lethal consequences worldwide. Gliomas are the most frequent brain tumors originated from glial cells of the central nervous system and most common and aggressive primary brain malignancy is called glioblastoma multiform (GBM grade IV astrocytoma). The median survival time for GBM patients is only 8-12 months. Despite the surgery, radiotherapeutic and chemotherapeutic interventions, the survival rates do not change remarkably. Thus, scientists across the world are still seeking for innovative therapies for gliomas. The presence of the blood-brain barrier (BBB) is an important factor in reducing the efficacy of chemotherapy. Temozolomide (TMZ), a DNA alkylating agent, which can penetrate the BBB due to its structure used as the most promising treatment for recurrent glioma.

In recent cancer researches, treatment of brain tumors has been shaped to reduce Hsp levels. The reasons for this are to show that there is a relationship between stress proteins and brain tumor malignancy, and that those proteins are effective in the proliferation and progression of tumor cells and that cancerous cells increasingly show resistance to treatment due to the increment in the expression levels of these proteins. Stress proteins have become an important focal point in developing new therapies due to the contribution to the suppression of these proteins' expression to the treatment. There are many studies in the literature that suppress the

expression of these proteins using distinct methods including targeting the TMZ agent and reducing the expression level of heat shock proteins. Recent research shows that cells can communicate through extracellular vesicles (EVs). Exosomes with spherical structures of 30-150 nm in diameter and surrounded by lipid bilayers have become the most studied group of the EV family. Exosomes have been indicated to have important roles in cancer improvement and can alter the tumor microenvironment with materials, which they can cargo (DNA, RNA, and proteins-some Hsps). Therefore, it is thought that cancer-associated exosomes and exosome surface HSPs can be a therapeutic target.

The main objective of this study was to evaluate the effects of TMZ on the expression of heat shock proteins in exosomes released from U87MG cells. At the beginning of the study, the effect of TMZ on cell viability was analyzed. According to the cytotoxicity analyzes, the agent was treated to the cells at concentrations that did not show any toxic or proliferative effect on the cells (100% cell viability) or that affected cell viability by less than 20% and 50% cell viability. After the exosome isolation was performed, the effect of TMZ application on both exosomal and cell-derived soluble protein profiles was demonstrated by 1-dimensional electrophoresis technique. The proteins were separated on the gels then transferred to the membrane by Western blotting technique and incubated with specific antibodies for target proteins. The most common exosomal marker proteins demonstrated the yield of the isolation by Western Blot and Scanning Electron Microscopy (SEM) images. Cell migration assay was carried out to explain the role of exosomes in metastasis.

According to the results of this study, it has been seen that TMZ at various concentrations in dose dependent manner treated to glioma cells increased the expression level of intracellular Hsp70, while the expression level of Hsp70 that is secreted by extracellular-exosomal pathway was decreased. It was determined that TMZ concentration was 750 μM, which the most increased Hsp70 expression in U87MG glioma cells and decreased expression of exosomal Hsp70. The results could be considered a promising approach in slowing cell migration and consequently slowing angiogenesis by decreasing the amount of extracellular Hsp70 by TMZ treatment.

These results indicate that the chemotherapy agent was administered to cells, perhaps becomes an effective therapeutic option for the brain tumors treatments. The data from this study are promising to the development of innovative therapies for the treatment of aggressive brain tumors with high fatality rates.

July 2019, 74 pages.

Keywords: Temozolomide (TMZ), glioma, exosome, Hsp70, cell migration.

1. INTRODUCTION

According to American Cancer Society Cancer Statistic Center, brain and other nervous system tumors are evaluated in the first 10 of the cancer types with fatal consequences worldwide, and 23.800 new cases of brain tumors are estimated in 2019 [1]. Gliomas are the most common brain tumors that derive from glial cells [2]. The most common and aggressive primary brain malignancy is glioblastoma multiform (GBM-grade IV astrocytoma). In GBM cases, cumulative cellular density, vascular proliferation, and necrosis have been observed more than other types of central nervous system cancers [3]. The average survival times for GBM patients are only 8-12 months [4]. These characteristics are the most important reasons for the aggressiveness of GBM and drug resistance. Despite surgery, radiotherapeutic and chemotherapeutic interventions, survival rates do not remarkable change.

In the GBM treatment, currently, multidisciplinary strategies are followed. Even if the maximum safe surgical resection is performed, it is necessary to be coupled with the administrations of a drug and/or radiation therapy [5]. The greatest challenge in the chemotherapy approach is the drugs that contain large molecules and more than 98% of small pharmaceutics cannot penetrate to the brain because of the blood-brain barrier [6]. Therefore, many scientists are still trying to develop better treatments for malignant gliomas.

Temozolomide (TMZ), a DNA alkylating agent, is the most promising chemotherapy agent for recurrent glioma. Thanks to the acceptable blood-brain barrier penetration, TMZ is used to treat patients with glioma longstanding. DNA might be damaged by the alkylating agents via the formation of different small or huge adducts based on nucleic acid [7]. This drug inhibits the development of cancer cells, slows cell growth and spreads to the body. Also, it is demonstrated that abundant levels of cell adhesion-related proteins were found in TMZ-treated EVs, suggesting an active role for the molecular cargo in EV-mediated communication [8].

In addition to temozolomide, Bevacizumab (Avastin), an FDA approved drug targeting Vascular Endothelial Growth Factor (VEGF), was mostly recommended for the treatment of brain cancer. Avastin is known as an anti-angiogenic drug, which inhibits the formation of new blood vessels required for invasiveness and tumor progression [9] but, anti-angiogenic therapy in the use of Bevacizumab has not been shown to improve for the patient' overall survival rate,

while, it has been demonstrated to continue to be effective in slowing tumor growth [10]. Moreover, low success rates have been observed in also carmustine (BCNU), lomustine (CCNU) used as single-agent or combined chemotherapy for high-grade gliomas' treatments. After all, surgery and radiotherapy continue to be the primary treatment. These agents are highly cytotoxic or insufficient tolerated and drug resistance arises.

Preclinical studies by the Cancer Research Campaign (CRC) have indicated that temozolomide has significant effectiveness and maximum safety profile in the glioma treatment. Temozolomide has been widely accepted as the first line chemotherapeutic drug in glioma treatment [11].

For a long time, scientists accepted that only hormones, cytokines or neurotransmitters provided intercellular communications. However, it is now well understood that cells can communicate via extracellular vesicles (EVs) [12]. Johnstone et al. [13] described exosomes that are the member of a huge EVs family, 30 to 150 nm in diameter, spherical structures surrounded by a lipid bilayer [14]. Exosomes became most and extensively studied group of the EVs family.

Recent researches highlighted a possible exosomal-mediated intercellular communication, which is involved in many different human diseases in many processes, for example, immune system signaling, angiogenesis, aging, proliferation, differentiation, and neurodegenerative disorders, cancer and AIDS [15]. Accumulative evidence indicates that exosomes have significant roles in cancer. The cells could convey information by signal transduction from the plasma membrane or internal elements through the extracellular vesicles. It has been stated that these tiny vesicles are capable to transfer nucleic acids and many oncogenic proteins to alter the activity of donor cells and play determinant roles in tumor growth, metastasis, invasion and drug resistance. Moreover, tumor-associated exosomes are mobile elements and tremendously contributed to in tumor angiogenesis by exosomal miRNAs, can directly deliver angiogenic proteins into endothelial cells or alteration the angiogenic function of endothelial cells [16]. Exosomes may also release a variety of tumor antigens, apoptotic proteins [17], tumor and tissue-specific peptides [18]. Stress proteins or heat shock proteins (HSP) are composed of a large family of proteins with conserved structures. They are classified into families by their molecular weights respectively, Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps (sHsp) [19]. HSPs were defined at first as a group of heat-induced proteins as well as many other stressors like ischemia, hypoxia, heavy metal and infections. They are also molecular

chaperones with important functions during stress, arbitrate protein folding, and retain protein structure and function. Their expression levels increase in many types of cancer, and HSPs suggests a poor prognosis in some types of cancer and response to treatment [20]. HSPs are shown to be overexpressed in gliomas, and they are known to be responsible for the resistance of gliomas against therapy. This is due to the ability of HSPs to play a key role in tumorigenesis through angiogenesis regulation, cell proliferation, migration, invasion, and metastasis [21]. These proteins are traditionally considered as intracellular molecules, but recent studies have shown that a new secretory pathway of exosomes emerged several years ago, and then they can be seen in extracellular localizations or in the blood [22]. Graner et al. [23] identified exosomes from cell lines of the brain tumor and analyzed their heat shock protein content as sources of extracellular heat shock proteins. Brain tumor exosomes displayed surface HSPs, particularly in GBM, high constitutive and inducible Hsp27 expression, aB-crystallin, Hsp70, and Hsp90 has been reported both in vitro and in vivo. In addition, have been GBM released exosomes may exhibit Hsp27, Hsp60, Hsp70, and Hsp90 [24, 25].

Exosomes are considered a potential contributor to cancer prognosis and became a novel approach. Therefore, it is considered that cancer-associated exosomes could be assessed as a therapeutic target. Exosome surface HSPs' studies will be of great interest. In order to explain the uncommon exosome membrane localization of stress proteins, the need for further studies.

In this thesis, we aimed to investigate the effect of TMZ on the expression of exosomal and cellular stress proteins levels' in U87MG human glioblastoma cells.

As a result of the TMZ treatment in glioma cells, the expression levels of stress proteins on the surface of exosomes were assessed. We determine the cytotoxic effects of TMZ on the U87MG cells by MTT method. TMZ was added to the control group cells and the experimental group cells at the determined concentrations. Three different doses of TMZ were applied to the cells according to MTT: 25 μM (non-toxic dose), 200 μM (20% toxic dose, IC_{20} value) and 750 μM (IC_{50} value). After 48 hours, the cells and exosomes were isolated from the cell culture medium. The effect of TMZ on the expression of Hsps, exosome marker proteins in both the cells and exosomes groups was evaluated by Western blotting analysis.

Our results showed that temozolomide reduces the level of extracellular Hsp while increases cellular Hsps in a dose-dependent manner. This can help bring a new approach to brain tumor

treatment. According to the migration (scratch) assay, it could be possible to say that temozolomide may slow the migration by decreasing Hsp70 secretion from the plasma membrane.

1.1. OVERVIEW OF CANCER

Cancer is the main cause of mortality in any region of the world and regardless of the level of human development in the 21st century [26]. It is estimated that cancer incidences and mortality burden worldwide will reach 29, 5 million by 2040 [27]. Accordingly, the number of studies on the diagnosis and treatment of cancer are expected to be raised over time. Therefore, the focus on developing new and effective treatments of cancer research has become extremely important.

Cancer is a common name that is resistant to anti-growth and apoptosis signals and is given to uncommonly divide common tumors. Cancer is called malignant when it can invade adjacent tissues, spread to distal parts of the body and create new blood vessels through angiogenesis and metastasis to produce secondary tumors in distant areas. This contrasts with a benign tumor, which continues to expand within the original tissue limits. Multiple cellular mechanisms are altered by malignant tumors that collectively fuel uncontrolled growth and block death signals [28].

Generally, brain tumors are called glioma and it is a sort of cancer, arises from glial cells, constitute the largest group of central nervous system tumors. There are a few types of glial cells (e.g. astrocytes, oligodendrocytes) and hence glioma may form divergent types of tumors. WHO categorizes and grades brain tumors based on their assumed cells of origin, i.e. ependymoma, astrocytoma, oligodendroglioma and mixed glioma [29]. Gliomas are in the group of astrocytic tumors, which arises from astrocytes, the most plentiful type of glial cells in the brain, composed of 81% of brain and nervous system malignancies [30].

According to American Brain Tumor Association Brain Statistics Analysis, there are more than 120 different types of primary brain and CNS tumors and about 32% of brain and CNS tumors are involved in the malignant category [31].

Glioblastoma multiform is one of the primary brain malignancy that is highly aggressive and has a poor prognosis. The mean survival times are less than 15 months, and the fatality rate is

over 50%. Because of the overriding progenitor (blast) cells of the embryonic nervous system (Glioblastoma), Percival Bailey and Harvey Cushing discovered the term almost a century earlier. The word in this context multiform was used to define the intro- and inter-tumor heterogeneity characteristic of GBMs [32].

By WHO 2007 classification, GBMs are included in the greater category of diffuse astrocytoma and are the most invasive Grade IV within this category. GBM is one of the best characterized brain cancers at the molecular level [33]. As a result of molecular analysis on glioblastomas, the researchers has asserted reported a linkage between GBMs and amplification of the epidermal growth factor (EGFR), tumor protein 53 (TP53) mutation, phosphatase and homologous tension (PTEN) mutation, genetic changes in chromosomal level (17p, 13q, 9p, 19, 10, 22q, 18q and 12q amplification showed that there are differences such as the expression of many gene families that are related to the adhesion, invasion, and angiogenesis of cancer cells. They also stated that the deficiency of heterozygosity (LOH) on chromosome 10 and 19q are by far the most common mutations in secondary GBM. For 30 years, several GBM cell lines, including U87 (>1900 citations in PubMed), U251 (>1100 citations) and T98G (>900 citations), have been used extensively and provide valuable data in this type of tumor researches [34]. In neuro oncological studies, the most widely employed human glioma cell line is U87MG [35, 36]. This cell line generally, brain tumors called glioma that is a sort of cancer, which derived from glial cells, form the largest group of CNS tumors.

U87MG cell line is a long-established and well-studied grade IV glioma, also regarded as the most malignant form of astrocytoma, glioblastoma multiform [36].

1.2. TEMOZOLOMIDE

Followed soon after the development of radiation therapy for glioblastoma treatment, researchers expressed that combining chemotherapy with radiation therapy augments the patient's survival rate [37]. Since reporting data on the efficacy of carmustine (BCNU), a compound that crosses the blood-brain barrier and is targeted to attack glioma cells for the first time, the activity of chemotherapy in the treatment of GBM has been improved by testing different drugs [38]. Today, the alkylating agent Temozolomide is a primarily preferential chemotherapeutic agent, which FDA approved for both low and high-grade astrocytic tumors treatment with its minor side effects [39]. The chemical name of TMZ is 3-methyl-4-

oxoimidazo [5, 1-d] [1, 2, 3, 5] tetrazine-8-carboxamide. It is stable at a pH less than 5, yet at a pH greater than 7 it is hydrolyzed to methyl hydrazine, 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide (MTIC, $C_6H_6N_6O_2$). As it is small (194 Da) lipophilic molecule, it is readily bioavailable as does not go through hepatic metabolism [40].

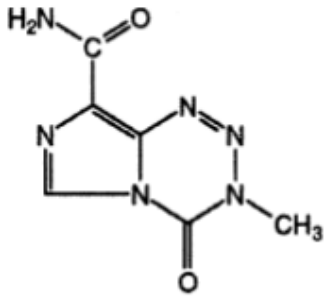

Figure 1.1: Chemical structure of temozolomide [41].

TMZ is an imidazotetrazine derivative of the DNA alkylating drug leading the cell cycle arrest at G2/M and bring along to apoptosis and permits the BBB penetration and a prodrug of the anticancer administered orally [42].

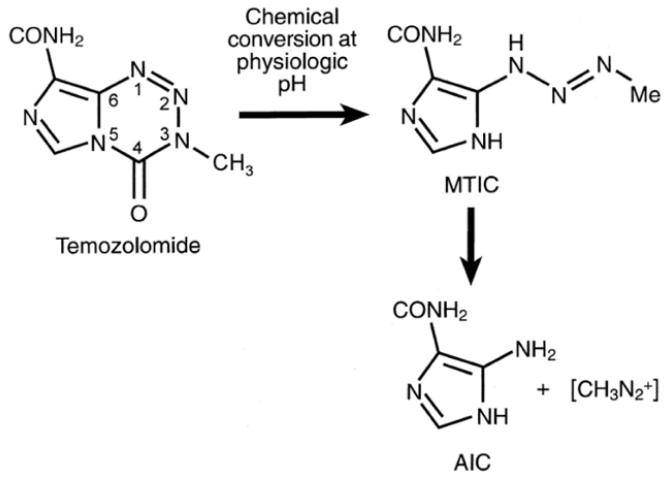

Figure 1.2: Chemical conversion of temozolomide [43].

The cytotoxicity of TMZ is mediated by it is turned into methyl diazonium cation by binding of methyl groups at N_7 and O_6 sites on guanines and the O_3 site on adenines in genomic DNA.

The methyl groups delivered to N_3 methyl adenine then the base excision repair (BER) which is a system high precision DNA repair system, could recognize thus N_7 methyl guanine does not produce any toxic effects.

Alkylation of the O_6 site on guanine cause to the insertion of a thymine in place of a cytosine reverse the methyl guanine which is recognized via DNA mismatch repair (MMR) system during subsequent DNA replication, MMR cut away and remove the impaired Thymine, yet cycles of insertion and excision trigger DNA double-strand break (DSB) and replication fork disintegrate. The cell cannot resist the attack and undergoes cell death [44].

However, the effectiveness of TMZ is inhibited by overexpression of DNA repairing enzyme, MGMT (O_6-Methylguanine DNA methyl transferase) that antagonizes the effect of TMZ and repairs the DNA damage. Absence of MGMT causes activation of MMR that results in double-stranded DNA break with subsequent cell death. Therefore, MGMT expression is a predictor of response to alkylating chemotherapy [45]. Efficacy of TMZ is limited by DNA repair protein MGMT [46], which comprises 207 amino acids (21 kDa). MGMT gene is located in Chromosome 10 at position 10q26. The MGMT promoter has a high CG content, (CpG islands) which have a tendency to bind abnormally methylated sequences. This suppresses the transcription factors, resulting in silencing of the gene [47, 48].

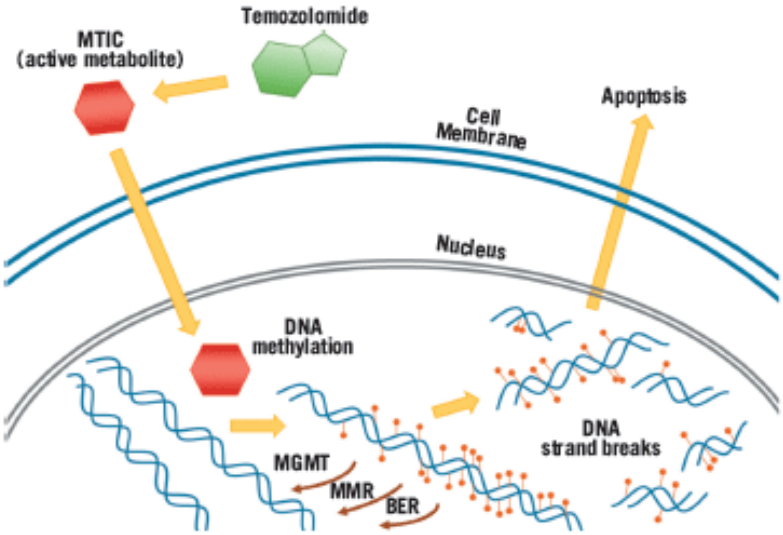

Figure 1.3: Mechanism of temozolomide [49].

1.3. EXTRACELLULAR VESICLES (EVs)

Cell to cell communication is a crucial process-taking place across all organisms. This information sharing occurs by either the bacterial gene transmission or soluble factors secretion [50].

The exosome was first discovered and published by Harding and Pan in 1983 [51]. They tried to find the path of transferring receptors between plasma membranes and reticulocytes. On this purpose, they cultured immature red blood cells, which reticulocytes with labeled transferrin receptors. They observed the labeled transferrin receptors were interiorized by the reticulocytes after that repacked into tiny vesicles as 50 nm size. At the early part of the research, these vesicles were assumed to lead to lysosome degradation as extracellular trafficking, consequently released into extracellular space from the mature blood reticulocytes. These vesicles can be found in many different body fluids like blood, cerebrospinal fluid, breast milk, breast milk, urine.

Subsequent investigations on this RNA shuttling mechanism confirmed that exosomes are biologically functional entities that can influence the phenotype and the behavior of recipient cells [52]. The BBB, which consists of endothelial brain capillary cells (BCECs), basal lamina and astrocytes, produce an important protective barrier that prevents the brain from possibly harmful substances in the blood circulation [53]. This physiological safety barrier as well noticeably inhibits the entry of so many potential drugs into the CNS, which is a major challenge for the delivery of brain drugs. The BBB cannot be penetrated by more than 98% of tiny molecule drugs and nearly 100% of proteins and peptides [54]. Scientists are also investigating the way of using those nano molecules as drug delivery vehicle. They are mobile particles; can enter the BBB [55].

1.3.1.1. The molecular composition of exosomes

The exosomal membrane proteins are localized similarly to the cell. All of the components such as lipids, proteins, and nucleic acids originated from the parental cells.

There are a set of exosome marker proteins to identify exosomes. These contain transport and fusion molecules, important proteins that are highly expressed during the secretion of exosome and include the Rab family of proteins.

Also GTPases, annexins, and the lipid raft-associated protein, Flotillin-1, is indicated as exosomal marker proteins, ESCRT complex and Alix as the accessory protein, tumor susceptibility gene 101 (TSG101), heat shock proteins (Hsp90a, Hsp70), integrins, and tetraspanin family as exosome identifiers CD63, CD81, CD9 and CD82 [56].

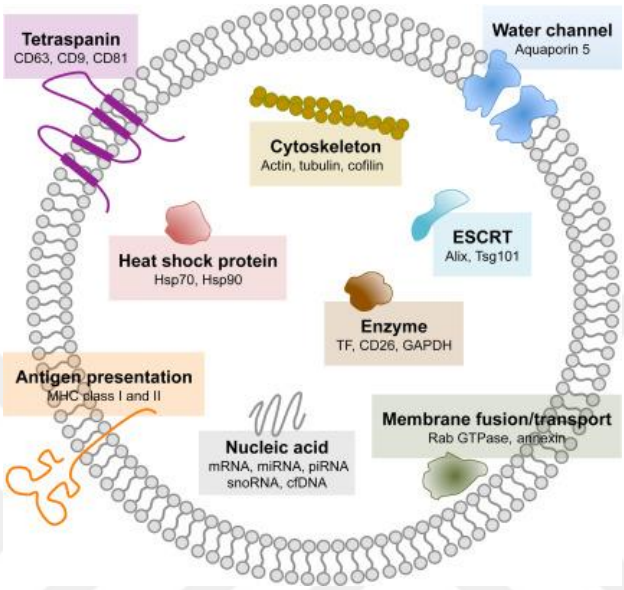

Figure 1.4: Composition of exosome [57]

1.3.1.2. Biogenesis and secretion

Exosomes, the smallest vesicle among EVs, range in size from 20 to 100 nm and are built by inward budding of the early endosomal membrane, forming intraluminal vesicles (ILV) within in the endosomes. When these endosomes carried exosome vesicles inside then are termed as multivesicular bodies (MVBs). Cocucci et al. [58] reported exosomes with the aid of electron microscopy that they were developed from MVBs that contain intraluminal vesicles (ILVs) and have an endocytic origin.

Exosomes are the member of a huge extracellular vesicles family with different sizes, which includes apoptotic bodies, microvesicles, oncosomes, and exosomes. It has been considered that all of these vesicles to involved in various biological process, particularly cell-cell communication by a serious impact on both the local environment and distant tissues. It also can carry cargo and express respectively DNA, RNA, and proteins among cells.

Numerously, based on the lattermost database of exosomes (http://www.exocarta.org/), there are 9769 proteins, 2838 microRNAs and 3408 mRNAs have been found in exosomes [59].

EVs are currently categorized into three types based on their biological function or biogenesis mechanism:

1) Apoptotic bodies, which are largest among EVs size ranges from 50 nm up to 5 μm and may contain DNA, RNA, and histone proteins. In case of apoptosis, they were released from dying cells, more than exosomes or MVs under particular circumstances, and then eventually removed by macrophages [50].

2) Microvesicles (50-1000 nm), that are produced by a plasma membrane and known as exfoliating vesicles that contain cytoplasmic cargo [60].

3) Exosomes are the smallest member of the EVs family ranging in size from 30 to 120 nm. Contains lipid bilayer membrane surrounded by cytosol and other components [61].

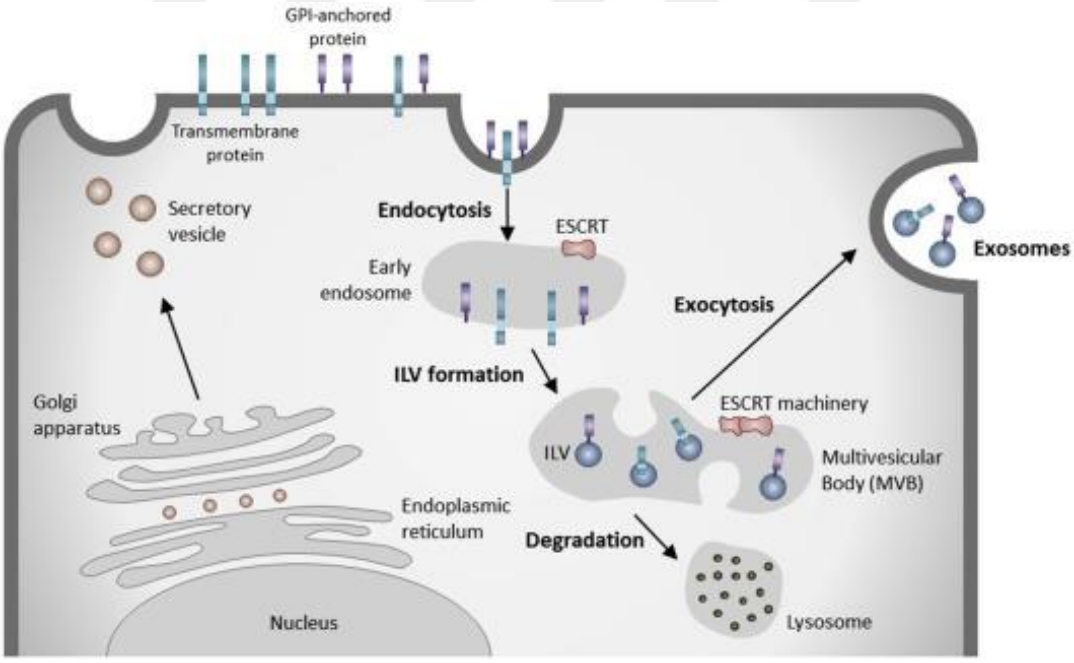

Figure 1.5: The biogenesis of exosomes [62].

Molecules shown to affect exosome biogenesis and release.

Exosomes, formed by inward budding of the plasma membrane occurs by early endosome differentiation.

Early endosomes budge into themselves and form a large number of intraluminal vesicles (ILV). The endosomal membrane is generating an inward budding by initiating with multiple elements: the tetraspanin proteins i.e. CD63 and CD9, as well as the most characterized pathway Endosomal Sorting Complexes Required for Transport (ESCRTs). The representation of the tetraspanin proteins come from their four transmembrane domains, that is highly elevated on the late endosomal membrane and provide some essential factors for exosome formation [63].

In order to release exosomes, several cellular steps need to be completed: These steps are the formation of intraluminal vesicles (ILVs) in Multivesicular Body (MVB), transport of MVBs to the plasma membrane and fusion of MVBs with the plasma membrane. Exosome biogenesis starts within the endosomal system; early endosomes mature into late endosomes or MVBs, and during this process, the endosomal membrane invaginates to generate ILVs in the lumen of the organelles [64]. Multivesicular bodies can either be directed to lysosomes where their content is degraded or transported to the plasma membrane for exosome release. In parallel, cargo is sorted to the emerging vesicles. There are four types of ESCRT [65]. ESCRT proteins on the surface of ILV, which form exosomes, consist of four subunits. ESCRT-0 is effective in the accumulation and recognition of ubiquitin proteins. While ESCRT-1 and ESCRT-2 provide membrane budding process and initiate ubiquitination of cargo proteins before the formation of ILVs. ESCRT-3 sparks the separation of the budding membrane. This mechanism is achieved by the formation of ESCRT-1, Tsg101, Alix and ESCRT-3 complex in the ILV membrane [66].

Various members of the Rab (Ras-associated binding protein group) family are effective in exosome secretion through the trans-Golgi network. Rab4, Rab5, Rab11, Rab27a, Rab27b, and Rab35 proteins are effective in exosome secretion in different cell types. Rab protein family moves the vesicle along the actin and microtubules on the plasma membrane with MVC; Provides fusion of the limiting membrane of MVC with the plasma membrane. This protein family is not only involved in exosomal secretion, but also in the progression of the endosome to the next steps. For example, Rab4 and Rab5 are effective in transforming the early endosome into MVC. Rab7 is one of the proteins that work in the lysosome orientation of MVC [67].

1.3.1.3. Roles of exosomes in cancer

EVs also play an important role in various tumor progression between cells. Tumor cells are capable to communicate with each other or among the various tumor microenvironment compartments to proliferate, spread and eventually affect adjacent cells. It has been considered; this communication with cancer cells has been linked to adherent junctions, cytokine interactions until recently [68]. This effect may vary with regard to the various GBM variants, based on the composition of the molecules that they secrete. It is also shown that mesenchymal cells are able to manipulate neighboring cells via miRNA participating [69]. In the past few years, extensive researches have focused on the EVs demonstrating their potentials to mediate cell communication with neighboring and/or distant cells [70].

According to the results of recent research, extracellular vesicles are known to have important roles in disease pathogenesis and tumor biology [71]. It was discovered several years ago that extracellular vesicles released by tumor cells have a role in tumor spread. These vesicles can alter cancer microenvironment in various ways and increase cancer progression; they can directly stimulate the formation of a previously metastatic tumor [72] and induce cell proliferation; they can promote angiogenesis, and remodel the extracellular matrix [73] and trigger immune escape by modulating T cell activity [74].

Al Nedawi et al. [75] indicated that a direct relation between extracellular vesicles and tumor invasion of healthy tissues. This study showed that mRNA expression of an activated mutated epidermal growth factor receptor (EGFRvII) in glioma cells increased the number of extracellular vesicles that could be detected even in the blood of tumor bearing mice, and that could lead to the production of (VEGF) in the neighbor tumor cells. Similar results have been stated by Skog et al. [72] that human primary glioblastoma shows that cell-mediated extracellular vesicle transfer EGFR with various miRNAs that promote tumor growth and angiogenesis.

Exosomes, the most well studied extracellular vesicles that trigger tumor spread, are secreted by cancer cells. Tumor derived exosomes have been indicated to be effective in tumor growth [73] cell adhesion [76] angiogenesis [77] and the development of drug resistance [78]. Researches confirm this information and it has been shown that exosomes released by human lung cancer cell lines (A549, HTB 177) and Lewis carcinoma (LCC) can induce the expression

of genes involved in angiogenesis such as VEGF, IL-8 and HGF [79]. In addition, Sheldon et al. 2010 [80] demonstrated that Dll4, an angiogenic factor in the content of exosomes, and its transport from U87 tumor cells to endothelial receptor cells. Dll4 transport led to changes in the cell phenotype: human umbilical vein endothelial cells (HUVECs) produced Dll4-containing exosomes. The introduction of U87 cells secreting Dll4-containing exosomes into xenograft mice has been reported to lead to the spread of blood vessels by branching, an increase in their sizes. It has been shown that exosomes can transfer their intracellular contents to communicate between both cancerous and healthy cells, thus it provides valuable knowledge for cancer biology.

1.4. HEAT SHOCK PROTEINS

Heat shock proteins (Hsp), also known as stress proteins, were originally discovered in 1962 as a protein group induced to the production of chromosomal puffs with respect to heat stress in the salivary glands of *Drosophila melanogaster* [81]. Subsequent studies have shown that Hsps are a family of evolutionary highly conserved proteins that exist across species. Most of these proteins are also known as molecular chaperones since they have protective roles in the cells. They assist protein folding and inhibit denaturation or misfolding/unfolding cellular proteins due to stress contributing causes [82].

This protein family represents about 2-3% of cellular proteins are present at low levels under normal circumstances; but considerably induced in response to induced by a wide variety of stressors, including heat shock, inflammation, oxidative / ischemic stress, toxins, heavy metal ions, radiation, environmental pollutants included chemotherapy. The increased expression of HSPs under stress conditions is usually regulated by heat shock factor 1 (HSF1) [83]. In the case of the stress conditions, HSF1 is phosphorylated and make homotrimers formations, then binds to heat shock elements (HSEs) located upstream of HSPs genes and activates the transcription of heat shock genes [84].

1.4.1. Stress Proteins Family and Functions

The nomenclature of human HSPs is based on the system proposed by the Human Genome Organization (HUGO) Gene Nomenclature Committee [85]. Heat shock proteins have been named depends on their molecular size that varies between 10-170 kDa: HSP 100, HSP90, HSP70, HSP60, HSP40 and sHSPs (s, small) [86].

The heat shock proteins of the HSP100 family [Hsp110 (human), Apg-1 (mouse), Hsp105, Osp94 (Hsp94), Grp170] have functions as molecular chaperones under physiological conditions. They are involved in rearrangement of proteins and dissolution of water insoluble protein aggregates in the cytosol [87].

HSP90 is the most studied member of the heat shock protein family that has been described as an anti-apoptotic protein that presents in most cells. It assists the conformational activation of many proteins under stress circumstances [88].

HSP70 family members have functions such as protection of proteins when they are exposed to stress, prevention of aggregation of folded proteins and transporting proteins. Hsp70 is secreted by a variety of cells via non-classical protein secretion pathways, including exosome pathways [89]. Extracellular Hsp70 shows immunomodulatory effects and plays a prominent role in immunomodulatory response to cancer cells [90].

HSP60 also chaperonin, is located in the cytosol and extracellular space that plays a crucial role in the transportation and mitochondrial proteins folding, and is indicated to be related to variable cancer types [91].

HSP40 family was reported that, also known as chaperone DnaJ, controls HSP70 function. It is shown that Hsp90 and the Hsp40–Hsp70 complex work together to promote the Akt pathway, well known and fundamental for the regulation cell cycle.

One of the members of the sHSP family, Hsp 27 is reported protein that was first characterized in response to heat shock protein as a chaperone, which assists the refolding damaged proteins. HSP27 also possesses a powerful antiapoptotic property. It also participates in cell migration, cell growth, differentiation, and progression of tumors [92].

1.4.2. The Role of Stress Proteins in Cancer

It has been proved that HSPs are highly expressed in cancer cells more than normal cells, allowing cancer cells to execute and maintain their cellular functions may require upregulation of HSP.

HSP110 in melanoma [93] and colorectal cancer [94], HSP60 in gastric cancer [95], Hsp70 in breast [96] and brain cancer [97], Hsp 40 in gastric [98], colorectal [99] and lung cancer [100]

Hsp90 in prostate cancer [101] and chronic myeloid leukemia [102], Hsp27 in brain tumor [103] and epithelial ovarian cancer [104] in such cancers has high levels of expression in various types of cancer have been found.

Various studies have shown that Hsps are important in the development of cancer based on the statement that over-expression of HSPs can induce carcinogenesis and drug resistance, and inactivation of Hsps can activate the apoptotic pathway.

Graner et al. [25] had previously reported Hsps on brain tumor cell surfaces and such HSP localizations have also been described. Members of the Hsp70 family group of stress proteins are actively secreted by different cells via non-classical protein secretion pathways, including exosome pathways have been studied for a long time [88]. In the literature, exosomal pathway secretion of stress proteins and exosomal-related HSPs have been shown to have an important role in the development of cancer [105]. Tumor cells that express Hsp70 on the plasma membrane, and they could secrete exosomes, which they have Hsp70 on their surface. As known, the protein component present in the structure of the exosomal lumen reflects the cytosol of the cell, hence the Hsp70 levels of exosomes from normal cells are expected to be less than exosomes of tumor cells with high cytosolic Hsp70 [106]. This information at least partially, in patients with head and neck squamous cell carcinoma tumors, were confirmed by studies showing that associated with the high expression of Hsp70 membrane [107].

Hsp70s are proteins that are significantly effective in the development of cancer and they are generally known to be overexpressed in cancer cells. Recent research has shown that a small proportion of the extracellular Hs70s are free Hsp 70s and these proteins are often released from dying cells [108].

It is discussed that Hsp70 can be secreted by tumor cells in two possible pathways. On the one hand, Hsp70 has been demonstrated to be secreted by dead cells; on the other hand, a large portion of the extracellular Hsp70 is released by living tumor cells by vesicular transport in a manner bound to a plasma membrane [109].

2. MATERIALS AND METHODS

2.1. CELLS AND CULTURE CONDITIONS

In this thesis, we used U87MG human glioblastoma cell line. The cells were provided from Animal cell culture collection of Istanbul University, Faculty of Science, Molecular Biology and Genetics Department. The cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C, and passaged every 3 days, before they reached full confluence. Experiments were performed using cells from passage number 3 to 10.

2.1.1. Subcultures of Cells and Media Used in Experiments

U87MG cells are sustained and subcultured in Dulbecco's Modified Eagle's Medium / High Glucose (DMEM/ High , Gibco 41966) medium L-Glutamine, 4,5 g/L D-glucose and pyruvate supplemented with 10% Fetal Bovine Serum (FBS, Gibco 10500), 1% antibiotic mixture (100 µg /mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B, Sigma-Aldrich A5955) and 1% "Non - Essential Amino Acids (NEAA, Gibco 11140, pH 7.4).

2.1.2. Conditioned Cell Culture Media for Exosome Isolation

For the exosome isolation, Exosome-free DMEM/ High Glucose medium was prepared by replacement of the normal FBS additive with Gibco™ Exosome- Depleted FBS, One-Shot™ format (Gibco A2720803) with supplemented 1% antibiotic mixture and containing 1% NEAA. All cells were cultured in a humidified incubator, set at 5% CO_2 and 37 ºC.

2.1.3. Cell Passaging

Subculturing of U87MG cell line was performed in the Laminar Air Flow sterile cabinet (Heal Force HFsafe-1200 version 2.01), subsequently followed these steps:

Glioma cells were maintained in high glucose DMEM growth medium with all additives (described in 2.1.1 and 2.1.2). After the cells reached to approximately 70% confluency, cells were subcultured by first discarding the old medium from T-25 cell culture vessels and the adherent cell monolayer was washed gently with Dulbecco's Phosphate Buffered Saline [10 mL, D-PBS, pH 7.2, Sigma-Aldrich D5652), 9.6 g/L of D-PBS was solved in dH_2O and sterilized by autoclaving (Nüve OT 012 Bench Top Unit Steam Sterilizer at 121 °C, under 1.2 atmosphere pressure, for 15 min] to remove serum residues that inhibit the activity of trypsin.

Cells were incubated in Trypsin/EDTA (2 mL, Sigma Aldrich) [0.2% (w/v) trypsin (Sigma-Aldrich T-4799) and 0.04% (w/v) EDTA (ethylenediaminetetraacetic acid, Invitrogen 15576) prepared in D-PBS was used after passing through a 0.22 μm sterile filter] at 37 °C for 3 minutes to remove the divalent cations which are effective in cell adhesion and detach the cells from the surface of the culture vessel. Under the action of the enzyme solution, the cells were kept in the incubator for about one minute until they detach from the culture dish. 5 mL (0.2 mL/cm^2) growth medium was added to a fresh T-25 flask to inhibit the activity of trypsin, and to separate the cells from each other and from the surface, they were attached to. Cell counting was performed with hemocytometer (Neubauer slide), the number of $2x10^5$ cells/mL U87MG cells were placed in a new culture dish and a total volume of 10 mL was completed with the medium. After the subculturing process, the cells were removed at 37 °C (Thermo Scientific, Heracell™ 150i) providing more than 90% relative humidity and 5% CO_2, and the subcultivation was repeated every 3 days to maintain cell cultures. Defined numbers of cells were then cultured in new cell culture vessels and placed in the incubator.

2.2. FREEZING AND STORAGE OF CELLS

Cells detached from the surface with trypsin-EDTA were centrifuged and the cell pellet was resuspended with 1 mL of medium. Trypsin was inhibited with at least twice volume of the medium. Cells were pipetted into a single cell suspension and transferred to a falcon tube. Two or three mL additional medium was added onto cell suspension. The cell suspension was centrifuged at 4100 rpm for 5 min to remove the supernatant. The cell number was counted by diluting with 900 μL growth medium. 100 μL of dimethyl sulfoxide (DMSO) and 900 μL of growth medium were added to cryotubes. The cryotubes were placed in the freezing container and stored in the freezer at -80 °C for use when needed.

2.3. GROWTH CURVE OF GLIOMA CELLS

In order to plot the proliferation curve of U87MG human glioma cells and calculate the generation time, cells with an initial number of $5x10^4$ and $1x10^5$ cells / mL were cultured in 24 well cell culture dishes. For 10 days, cell counting was performed every day at the time the culture was started. For cell counting, 10 μL trypan blue, a dye able to penetrate through the membranes of the dead cells, was prepared with cell suspension at a ratio of 1:1 (v:v) [Merck 111732 was prepared in 0.5% (w/v) D-PBS]. The number of live cells was determined by counting cells that were not stained. Counts were performed in 6 replicates. Nonlinear

regression (curve fit) was plotted by using the data obtained in GraphPad Prism® 5.01. The lag, log and stationary phases of the cells were determined by the semi-logarithmic multiplication curve. The generation numbers and times of the cells were calculated according to Equations 3.1 and 3.2.

(Equation 3.1) $n = (\log N_2 - \log N_1) / \log 2$

n: Generation number

N_2: Number of cells at the end of the logarithmic division stage

N_1: Number of cells at the beginning of the logarithmic division stage

(Equation 3.2) $St = (T_2 - T_1) / n$

St: Generation time

T_2: End time of the logarithmic division stage

T_1: Start time of logarithmic division stage

2.4. CYTOTOXICITY ANALYSIS

The cytotoxic effects of the temozolomide (TMZ, Sigma-Aldrich T2577), applied on U87MG cells were determined by MTT colorimetric method following the procedure recommended [110]. The principle of this method is that living cells convert MTT (3-(4, 5-dimethylthiazol-2, 5-diphenyltetrazolium) into water-insoluble formazan crystals with mitochondrial dehydrogenase activity and these crystals are dissolved in DMSO and measured at 540 nm in spectrophotometer.

In order to determine the cytotoxic effects of the TMZ cells were cultured in 96-well plates at a density of 8000 cells/cm^2 in a total volume of 200 µL of DMEM/ High Glucose medium and the cells were incubated in an incubator at 37 °C, providing more than 90% relative humidity and 5% CO_2 for 24 hours. At the end of the incubation, the culture medium was removed and TMZ was applied to the cells in different concentrations. Cells were incubated for 24 hours in a 5% CO_2 incubator at 37 °C. After removing the medium from the cells attached to the culture dish, the control group cells were cultured on the DMEM/ High Glucose medium. Drug was

applied to the cells were prepared by dissolving stock solutions at a concentration of 100 mM in DMSO (99.9%, Merck 116743). Different concentrations of temozolomide (Sigma-Aldrich T2577) (1, 10, 50, 100, 250, 500, 750, 1000 and 1500 μM) were tested on cells. The toxic effects of the drug were evaluated after 48 hours.

Cells were then incubated for 48 hours and cell viability was measured with MTT (Sigma M5655) stock solution (5 mg/mL, prepared in D-PBS) was added to the cells and incubated in the incubator for 4 h in dark. 150 μL of DMSO was added to each well at the end of incubation to dissolve the formazan crystals, and the culture flask was shaken at room temperature until the solution became homogenous (150 rpm, Heidolph Unimax 1010). Absorbances was measured at 570 nm by microplate reader (EON, BioTek Instruments Inc.), and the absorbance values were compared according to the control and the viability of the cells was determined as % viability by using Equation 3.3.

(Equation 3.3) $\%\ \text{Viability} = (AE / AC) \times 100$

AE: Absorbance of experimental group

AC: Absorbance of control

The experiments were carried out at least 6 replicates and according to the results of the analysis, the concentrations of the drug used in the thesis to be applied to U87MG cells were determined. Finally, dose response curves were plotted and IC_{50} values were calculated by using the Graph Pad Prism 7.00 software.

2.5. TEMOZOLOMIDE TREATMENT

Applications of TMZ were carried out in T-175 culture dishes. According to the cell count results in the hemocytometer, an appropriate amount of cells were taken and placed in culture dishes (see Table 2.2) and monolayer cultures were formed. Applications of TMZ were carried out in T-175 culture dishes. Cells were incubated in a 37 °C incubator for 24 hours, providing more than 90% relative humidity and 5% CO_2. TMZ were used in non-toxic doses determined according to the results of cytotoxicity analysis (MTT test). After 24 hours of incubation, the old medium on the cells was removed and replaced with a fresh medium containing exosome depleted serum and the TMZ at the determined concentrations were added to the experimental group cells. Control group cells were treated with only DMEM medium containing exosome

depleted serum. Three different doses of TMZ according to MTT results were applied to the cells: 25 μM (non-toxic dose), 200 μM (20% toxic dose, IC_{20} value) and 750 μM (IC_{50} value) for 48 hours.

Table 2.1: Experimental groups used in this thesis, short descriptions and representations.

Experiment Group Number	Description of Experimental Group	Abbreviation
1	Temozolomide untreated group = Control	C
2	Non-toxic effect temozolomide treated experimental group	IC_0
3	20% < cell viability effect temozolomide treated experimental group	IC_{20}
4	Toxic effect temozolomide treated experimental group	IC_{50}

Table 2.2: Number of cells used in the thesis.

Cell culture flask	Area (cm^2)	Cultivated Cell Count
96 well plate	0.34	$17,5 \times 10^4$
6 well plate	9.5	2.8×10^5
24 well plate	1.9	9×10^4
T-25 culture flask	25	7.35×10^5
T-175 culture flask	175	2×10^5

2.6. HARVESTING OF THE CELLS

After drug administration to the culture for 48h, followed the cell harvesting protocol: the medium was collected from the surface of the attached cells to use to downstream process later, and then they were washed with D-PBS (0.2 mL/cm^2) gently. In order to detached cells from surface and each other, Trypsin/EDTA was added to cells. The culture flasks were kept in the incubator for about one minute until the cells were detached from the culture vessel by the action of the enzyme solution. Then the medium was added (T-175 cm^2 / 10 mL) into the total cell suspension and it was taken into a sterile tube at RT (25 °C) and centrifuged, at 3000 *g* 5

min (Nüve, NF 400). After this process is completed, the supernatant (medium) was discard; cell debris was suspended again in cold D-PBS and centrifuged under same conditions. In order to remove all the medium residues washing step was repeated two more times. Cells were kept at -80 °C until they will be needed .

2.7. ISOLATION OF WATER-SOLUBLE CELLULAR PROTEINS

250-300 µL cold (4 °C) lysis buffer [0.02M Tris-HCl (pH 6.8, AppliChem A3452), 0.04% (w/v) EDTA, 1% (v / v) Triton X-100 (Merck 108603), EDTA free protease inhibitor cocktail (1 tablet/50 mL Roche 11873580), 1 mM PMSF (phenylmethylsulfonyl fluoride, AppliChem A0999)] was added and the pellet was resuspended. Both the syringe and the ultrasonic water bath were used to completely disrupt the cells. Cells were subjected to mechanical force with the help of 1 mL insulin syringes and then they were shredded by ultrasonic water bath (WiseClean-Daihan WUC-D10H) and ice. This step continued with repeated periods (3-5 cycles) for each sample. After disruption, the homogenate was centrifuged at 20000 *g* for 20 minutes at 4 °C (Sigma 3-30K) and the water-soluble proteins contained in the supernatant were taken into a fresh tube and stored at -70 °C for use in Western blot analyzes [111].

2.8. EXOSOME ISOLATION PROTOCOL

2.8.1. Collection and Filtration of Supernatant for Exosome Isolation

After drug treatment, supernatant with exosome depleted serum was collected from 175 cm^2 flasks at the end of 48 hrs. Exosome purification from cell culture media was conducted as described in section 2.1.2.

1. All conditioned medium was removed and transferred to a sterile tube and then filtered through by using a 0.45 µm Millex® AA Filter Unit (MF-Millipore, SLAA033SB) into new sterile fresh tube.

2. Once filtered medium, 12 mL of CM for each step, was clarified by serial centrifugation with Centrifugal Filter (Amicon ultra-15 device-50k Merck) at 5000 *g*, 15 min at 4 °C (Eppendorf Centrifuge 5810 R). Flow through was discarded. The supernatant was taken to a fresh tube. This step was repeated until all the media was filtered through. Clarified media was immediately used in the exosome isolation by using the exoEasy

Maxi Exosome Isolation Kit (membrane affinity spin columns for CM, Qiagen, cat. no. 76064).

3. Medium and XBP (exoEasy Maxi Kit Qiagen) buffer was mixed at 1:1 ratio in sterile tubes by gently inverting the tube 5 times.
4. The sample and XBP mix was added onto “exoEasy Spin column” then centrifuged at 500 *g*, for 5 min at 4 °C.
5. Flow through was discarded and the column was washed with XWB (ExoEasy Maxi Kit Qiagen) and centrifugated at 5000 *g*, for 5 min at 4 °C.
6. ExoEasy Spin Column was placed into a fresh collection tube. Then elution buffer (Buffer XE ExoEasy Maxi Kit Qiagen) was added onto “exoEasy Spin Column”. After 1 min incubation at RT, centrifuged at 5000 *g*, 5 min 4 °C .
7. 400 µL of Buffer XE was reapplied to the exoEasy spin column membrane, incubated for 1 min at RT. Final centrifugation of the elute was performed at 5000 *g*, 5 min.
8. The eluate was collected and transferred to an Eppendorf tube.
9. RIPA Buffer with 10X ratio [25mM Tris-HCl (pH 7.6, AppliChem A3452), 150mM NaCl (Merck 106400), 1% (w/v) NP-40 (Nonidet P40, AppliChem A1694), 1% (w/v) Sodium deoxycholate, 0,1% SDS AppliChem A2263)] and EDTA free protease inhibitor cocktail from 25X stock solution were added into eluate volume.
10. The elute was centrifuged at 12 000 *g*, 10 min at 4 °C (Sigma 3-30K)
11. Supernatant-exosome suspension was collected, aliquoted as 4 x 1.5 mL micro centrifuge tubes, and stored at -20 °C.

2.9. SCANNING ELECTRON MICROSCOPY (SEM)

Scanning Electron Microscopy (SEM) images were obtained from Istanbul University, Faculty of Science, Physic Department, Advanced Lithographic Techniques Laboratory. The pellets containing exosomes isolated from cell culture supernatant of U87MG cells (see section 2.8.1) and dried by a freeze dryer (Martin Christ) overnight. After the samples dried then they were transferred onto carbon coated electron microscopy grids. Exosome sizes were analyzed at 10.0 kV by using the EM (FEI VERSA 3DLOVAC) images.

2.10. DETERMINATION OF PROTEIN CONCENTRATIONS

SMART^{TM} BCA (bicinchoninic acid) Protein Assay Kit (iNtRON Biotechnology) was used to determine the protein concentration of the cell extracts and isolated exosome samples (see section 2.8.1).

The principle of this assay relies on the formation of a Cu (II) ions protein complex under alkaline conditions, followed by reduction of the Cu (II) ions to Cu(I) ions. The amount of reduction is proportional to the amount of protein present. BCA (water-soluble stable compound) forms a purple complex with Cu (I) in alkaline environments is measured by a spectrophotometer at 562 nm.

Protein concentration was determined according to the manufacturer's protocol. Bovine Serum Albumin (BSA) supplied from the producer was diluted following the recipe. Protein standards were prepared by diluting with dH_2O ranging between 0-2000 μg/mL (25, 125, 250, 500, 1000, 1500 and 2000 μg/mL). The samples were then diluted with 5 times dH_2O (blank, solvent of samples lysis buffer). 200 μL BCA reagent was [prepared by mixing the reagent A and reagent B solutions in the kit at a ratio of 50:1 (v:v)] added to each well of 96-well microplate. 25 μL of the diluted samples and blank sample were added on to the BCA reagent. The microplate was shaken on an orbital platform shaker (Heidolph Unimax 1010) for one minute, then incubated in an incubator (Thermo Scientific, Heracell™ 240) at 37 °C for 30 minutes. Finally, the absorbance of the colored product was measured by using (EON, BioTek Instruments Inc.) at a wavelength of 562 nm. All samples and standards were evaluated as 3 replicates.

A standard curve was generated using the concentration and absorbance values of the BSA standards. Protein concentrations were calculated by placing the absorbance values of the protein samples in the linear equation of the standard curve.

2.11. SDS-PAGE

The method proposed [112] was used to separate water soluble cellular proteins by denaturing gel electrophoresis (SDS-PAGE).

The system actually consists of two gels: a separating gel in which proteins are resolved depends on their molecular weights (MWs). A stacking gel in which proteins are concentrated previously to entering the separating gel. Differences in the compositions of the stacking gel,

separating gel and electrophoresis buffer make a system that is capable of finely separating proteins.

Mini gel electrophoresis system (Mini-PROTEAN® Tetra Cell, Bio-Rad) was used for the analysis of water-soluble proteins. Glazing (1.0 mm spacer plates and short plates, combs (1.0 mm) and gaskets (gaskets) were cleaned with 70% ethanol (EtOH) and completely dried before the gel was prepared. The gel cassette was assembled in accordance with the manufacturer's product manual.

Firstly, pH 8.8 Tris-HCl, dH_2O, acrylamide solution, SDS and APS in the amounts as given in the Table 2.3 were mixed for the preparation of 10% separating gel, the ingredients are mixed gently, and kept in the ultrasonic water bath for 5 minutes to remove the dissolved oxygen from the mixture. After adding TEMED (Fluka 87689), the gel mixture was mounted between two glasses (1 mm) with the help of the syringe to fill 4/5 of the prepared cassette. The gel surface was sealed overlaid with about 1 mL of dH_2O to prevent contact with the oxygen, which inhibits polymerization, and to ensure that the top of the gel was flat. After the polymerization was completed (about 1 hour), the surface water was removed, and a 5% loading gel was prepared and poured onto the polymerized separation gel with the help of the syringe. A comb was inserted carefully and quickly on the top of the cassette to generate wells in which samples were loaded. Once the stacking gel has polymerized (about 30 minutes), the binder clips, spacer, and comb were removed gently from the gel assembly. After polymerization, the wells were washed with ultra-pure water. The gel cassette was placed in an electrophoresis tank filled with 1X tank buffer.

Table 2.3: The solutions for SDS PAGE.

Solutions	Ingredients
30% Acrylamide Stock Solution	Acrylamide (Sigma-Aldrich A3553) ..... 30 g Bis-Acrylamide (Sigma-Aldrich M2022)..... 0.8 g Fill up to 100 mL with dH_2O
1.875 M Tris-HCl Buffer (pH 8.8)	Trizma-Base (Sigma-Aldrich T1503) .....45.43 g pH was adjusted to 8.8 with 3N HCl Fill up to 100 mL with dH_2O
0.6 M Tris-HCl Buffer (pH 6.8)	Trizma-Base .....14.54 g pH was adjusted to pH 6.8 with 3N HCl Fill up to 200 mL with dH_2O.
10% Sodium dodecyl sulfate (SDS)	SDS (AppliChem A2263) .....10 g dH_2O Fill up to 100 mL with dH_2O
10% Ammonium persulfate (APS)	APS (AppliChem A2941)..... 0.1 g Fill up to 1 mL with dH2O Freshly prepared.
1X Running Buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3)	Trizma-Base.....…3.03 g Glycine (AppliChem A1067) ..... 14.4 g SDS.....1 g Fill up to 1 L with dH_2O
2X Sample Loading Buffer	0.6 M Tris-HCl Buffer (pH 6.8)..... 5 mL SDS..... 0.5 g Sucrose (Sigma-Aldrich S0389).....14.54 g β-Mercaptoethanol (AppliChem A1108) ..... 0.25 mL Bromo-phenol blue (0.5% (w/v) stock, Merck 108122).....5 mL Fill up to 50 mL with dH_2O

Table 2.4: Separating gel and stacking gel contents (required volumes for 1 gel).

Gel Contents	Separating Gel (10% Acrylamide)	Stacking Gel (5% Acrylamide)
Tris-HCl (1.875 M, pH:8.8)	1 mL	-
Tris-HCl (0.6 M, pH:6.8)	-	250 µL
dH_2O	2.262 mL	1.875 mL
%30 Stock Acrylamide Solution	1.662 mL	337.5 µL
%10 Sodium Dodecyl Sulfate (SDS)	50 µL	25 µL
%10 Ammonium Persulfate (APS)	25 µL	12.5 µL
TEMED	1.75 µL	3.5 µL

The samples were mixed with 2X sample buffer in a ratio of 1:1 (v:v) and boiled for 5 minutes to fully denature the proteins. The samples were loaded to the gel with 30 μg of protein in each well. The first lane of the gel was filled with a protein marker mixture (Pierce® Blue Prestained Protein MW Marker Mix, Thermo Scientific 266121). Electrophoresis was performed at constant voltage (200 V) and separation was continued until the band of tracking bromophenol blue reached the lower edge of the gel (about 40 minutes).

2.12. COOMASSIE BLUE STAINING

Subsequent to electrophoresis, Coomassie brilliant blue staining method was used to make the protein bands visible on the gel. After the SDS-PAGE has been completed, the gel was placed into a plastic container which is a resistance to alcohol and acid. Then the gel was shake in the staining solution [0.05% (w/v) Coomassie brilliant blue R-250, (Merck 112553) was prepared the mixture of 50:10:40 ratio MeOH (Methanol, Sigma-Aldrich 24229) : AcOH (Acetic Acid, Sigma-Aldrich 27225):dH_2O] at RT for 2 hours at room temperature necessary to visualize the bands of interest.

In order to remove excess dye, the gel was soaked in destaining solution [MeOH: AcOH: H_2O prepared in volume ratios of 5: 7: 88] for 12 hours via shaking 50 rpm on the shaker. The solvent was changed frequently, and the washing process was repeated until the background of the gel was completely destained. The bands formed in the gel were visualized using ImageLab 5.2.1 software in the ChemiDoc MP (Bio-Rad) imaging system.

2.13. WESTERN BLOT ANALYSIS

All of the buffers and their contents used in Electrotransfer, Western blotting and membrane stripping methods are given in Table 2.5, primary and secondary antibodies used in immunological analysis, their sources, and the organisms and dilutions are given in Table 2.6.

Proteins, which were isolated from cells and exosomes separated in the 10% gel that contains polyacrylamide, were transferred to the polyvinylidene difluoride (PVDF) membrane by the electroblotting method [Wet/ Tank Blotting System (Bio-Rad)]. In this method, two pieces of filter paper and one piece of PVDF membrane whose dimensions are the same as that of the gel were cut. Firstly, membrane was laid in 100% methanol for 30 seconds; after that, the membrane, a set of fiber pads, filter paper and the SDS-Gel were soaked in transfer buffer for 20 minutes.

The order from cathode (black side) to anode (white side) is as follows: the cassette was placed with the black side down, prewetted fiber pad was placed on the black side, a sheet of filter paper on the fiber pad, the equilibrated gel was placed on the filter paper, the prewetted membrane on the gel. The sandwich was completed with a piece of filter paper on the membrane; the last fiber pad was added on the filter paper. After the air bubbles between the layers were removed with a roller, the cover of the cassette was closed white latch the placed in the module firmly and carefully not to move the membrane and gel sandwich. The cassette and the cooling unit were put into tank. The transfer chamber and tank were filled with freshly prepared transfer buffer then cover the lid and connected the Wet/ Tank Blotting System with the power supply, set the voltage and then start running for 90 minutes. After the transfer, the sandwich was dissembled; membrane was removed from the stack and stained with Ponceau S to confirm that the transfer has been successful.

Table 2.5: Buffers and their contents used in electro transfer, Western blotting and membrane stripping methods.

Solutions	**Ingredients**
1X Transfer Buffer	Trizma-Base..... 3.03 g Glycine..... 14.4 g Fill up to 1 L with dH_2O
5% Blocking Buffer	Non- Fat Skimmed Milk Powder (Fluka 70166) .....5 g Fill up to 100 mL with 1X washing solution
1X Washing Buffer (1X TBST-Tween-20)	Trizma-Base (20 mM, pH: 7.5)..... 2.42 g NaCl (150 mM) (Merck 106400) ..... 8.77 g Tween-20 (0.1%) ..... 1 mL Fill up to 1 L with dH_2O
Stripping Buffer	10% SDS.....20 mL 0.5 M Tris-HCl (pH: 6.8)..... 12.5 mL Ultrapure water..... 67.5 mL 2-mercaptoethanol ..... 0.8 mL

Table 2.6: Primary and secondary antibodies used in immunological analysis (sources and dilutions).

Antibody	Mono/Polyclonal	Manufacturer company	Code	Source	Dilution
Hsp 70	Monoclonal	Enzo Life Sciences	ADI-SPA-810-F	Mouse	1:1000
Alix	Monoclonal	Santa Cruz	sc-53540	Mouse	1:200
TSG101	Monoclonal	Novus	NB200-112	Mouse	1:500
GAPDH	Monoclonal	Thermo-Invitrogen	MA5-15738	Mouse	1:2000
CD81	Monoclonal	Novus	NB100-65805	Mouse	1:500
CD63	Monoclonal	Santa Cruz	Sc-5275	Mouse	1:200
Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	Polyclonal	Enzo Life Sciences	ADI- SAB-100-J	Goat	1:2500

Following the transfer, the membrane was incubated for 2 hours at room temperature in 5% (w/v) skimmed milk powder (Fluka 70166) prepared in TBST [TBS containing 0.1% (v/v) Tween-20] containing 5% blocking buffer to block nonspecific antibody binding the membrane.

After blocking, the membrane was incubated with primary antibody which was prepared in appropriate proportions in blocking buffer at 4 °C with gentle shaking overnight. The antibodies were diluted in skimmed milk solution as shown in Table 2.6.

When the primary antibody incubation was completed, the membrane was washed 4 times with 1X wash buffer TBST and shaken at low speed for 15 minutes each to remove antibody residues. The membrane was then incubated with a secondary antibody, conjugated horseradish peroxidase (HRP), for 2 hours gentle shaking at room temperature and subsequently washed with 1X TBST four times for 15 min before chemiluminescent detection. The reporter enzyme HRP, which is conjugated to the secondary antibody, used for detection. In the presence of HRP oxidizes the compound luminol is oxidized in a chemical reaction that emits light (chemiluminescent). A reagent and B reagent solutions in the kit were mixed in a ratio of 1:1 (v:v). The detection of a chemiluminescent signal obtained after adding the luminol containing Super Signal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) to the membranes. In the dark room, under red light, this mixture was added to the membrane and allowed to stand for 5 minutes. After this process, the mixture on the membrane was carefully

removed and the membrane was placed between the two transparency papers without any air bubbles and placed on the imaging device (ChemiDoc MP, Bio-Rad). The image obtained from the membrane was analyzed by using ImageLab 5.2.1 software.

In order to normalize the analyzed membranes, bound antibodies to target protein imprinted membranes were removed and the membrane was made suitable for re-blotting. Stripping aims at removing bound primary and secondary antibodies from a membrane. In order to be able to perform this process, firstly stripping buffer was heated to 50 °C. After the hot buffer was poured over the membrane, the membrane was incubated at 50 °C for 45 minutes. Next, the membrane was then washed with ultrapure water 4 times with shaking at low speed for 15 minutes. Then the membrane taken into a clean container then it was rinsed with TBST buffer 4 times for 15 minutes at low speed and rinsed again. At the end of this procedure the membrane became suitable for blotting. For normalization, the stripped membrane was incubated for overnight at 4 °C with GAPDH antibody. At the end of the incubation, the membrane was washed with TBST buffer 4 times for 15 minutes with low rpm. GAPDH bands were then displayed using the Super Signal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific) Western Blotting Detection System and normalization of the protein bands in the membrane was performed by normalization calculations in ImageLab 5.2.1 software.

2.14. CELL MIGRATION ASSAY: *In vitro scratch assay*

U87MG cells were cultured in 24-well plates at a density of 15000 cells/cm^2 in a total volume of 200 µL of DMEM/ High Glucose medium and the cells were incubated in an incubator at 37 °C, providing more than 90% relative humidity and 5% CO_2. After 24 hours, the cell monolayer was gently and slowly scratched a straight line with 1 ml pipette tip across the center of the well. Second straight line was scratched perpendicular to the first line to make a cross on the surfaces of the monolayer cells in each well. After the formation of the scratch, the cells were washed with PBS to remove the detached cells from the surface and imaged under light microscopy. Control group cells were treated with only DMEM medium; the experimental group cells were treated with DMEM medium with exosome depleted serum containing temozolomide at 25, 200 and 750 µM concentrations. At 24 and 48 h, the cells were imaged under the light microscopy. In order to document the results, photos were taken on invert microscope (Nikon Eclipse Ti-E) at 0, 24, 48 hours after, the gap distances were quantitatively evaluated using Image J software.

2.15. STATISTICAL ANALYSIS

All numerical data of cytotoxic and immunological analyzes performed in the scope of the thesis were evaluated statistically. Arithmetic means and standard deviation (SS) were calculated by using GraphPad Prism® 7.01 program. The significance of the differences between the groups was determined using Student's t-test or one-way or two-way ANOVA (analysis of variance)-followed by the Dunnett or Tukey post hoc tests. The significance limit was accepted $P < 0.05$.

3. RESULTS

3.1. GROWTH CURVE OF GLIOMA CELLS

In the cultures with an initial cell number of $5x10^4$ and $1x10^5$ cells/mL, the growth curve of the U87MG cells were obtained by cell counting for 10 days from the beginning of the culture. The growth curve is shown in Figure 3.1. According to this graph, it was determined that the cells enter into the logarithmic phase on the 1st day in both cultures. Cells initiated with $5x10^4$ cells/mL entered stationary phase on 5th day of culture, while cells initiated with $1x10^5$ cells/mL entered in the 3rd day of culture.

According to the graph, the generation time of U87MG cells was approximately 17 hours. This result is consistent with the literature [125].

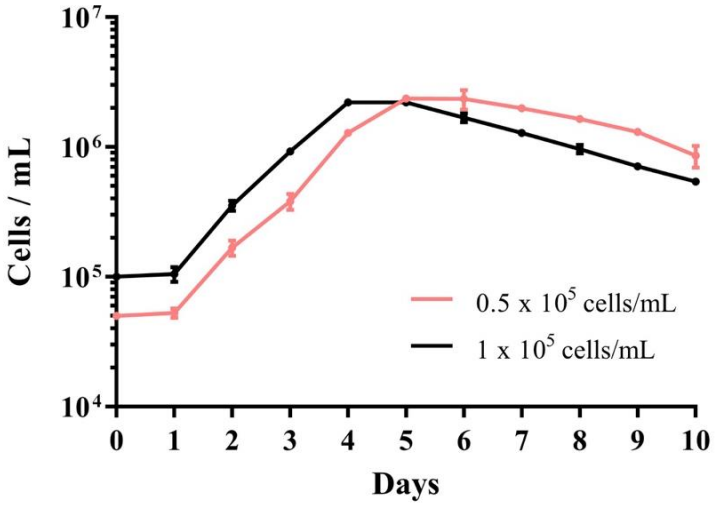

Figure 3.1: Growth curve of U87MG cells with the initial cell number of $0.5x10^5$ and $1x10^5$ cells/mL (vertical bars show standard deviation values).

3.2. CYTOTOXIC EFFECT OF TEMOZOLOMIDE

In order to evaluate the cytotoxic effects of temozolomide on U87MG cells and determine IC_{50} (half maximal inhibitory concentration) value, MTT analysis (viability test) was performed with 24 replicates. After 24-hours later, cultures were treated with media containing 0 (representing the control group), 1, 10, 50, 100, 500, 750, 1000 and 1500 µM. Temozolomide concentrations. The data obtained from the MTT analysis are given in Figure 3.2. According to the MTT results, the control group (0 µM) was considered as 100% alive, and the consistency

between the replications in the experimental groups were determined by one-way ANOVA test followed by Dunnett's post hoc test. The concentrations of TMZ applied to the cells, which reduced the number of cells, by 50% in IC_{50} at 48 hours were calculated by using a sigmoidal dose-response curve in GraphPad Prism (version 7.00).

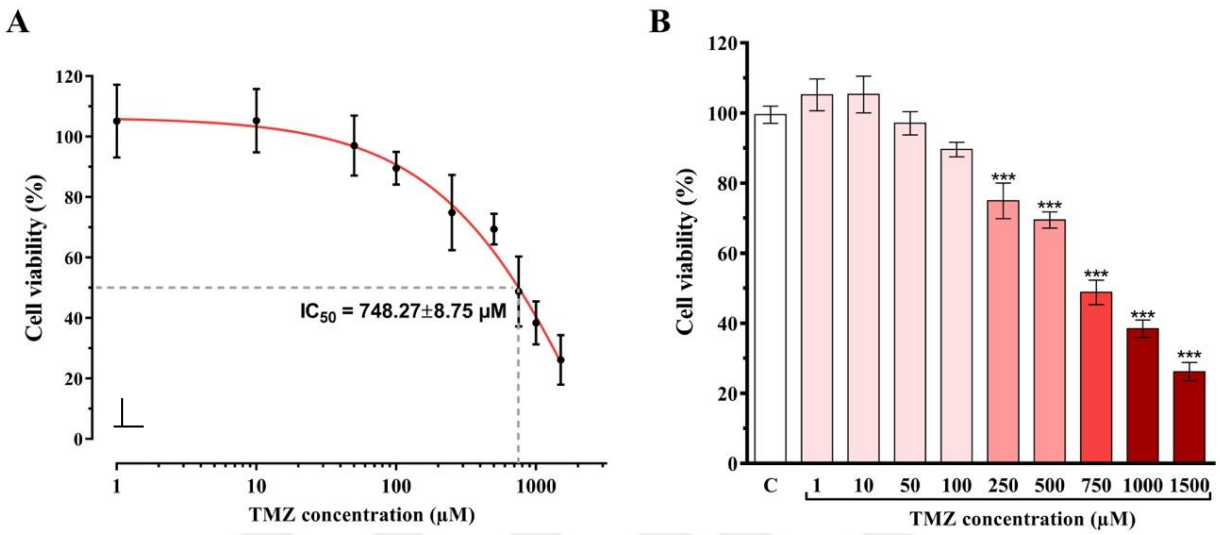

Figure 3.2: Effect of temozolomide on the viability of U87MG cells. **A)** The dose-dependent curve of temozolomide on U87MG cell viability (R^2 = 0.872), **B)** Effect of temozolomide on the viability of U87MG cells at different concentrations (*** *P* <0.001, vertical bars indicate standard deviation values).

MTT results showed that (Fig. 3.2) TMZ treatments in U87MG cells induced cell death in a dose-dependent manner. At low doses of TMZ (1 and 10 µM) were showed proliferative effects, and at high doses of TMZ (1000 and 1500 µM) inhibited cell viability by less than 50%. As a result of analyzes, the temozolomide concentration which reduced the number of viable cells by 50% was found as 748.27 µM (IC_{50} = 748.27 µM). 25 µM of temozolomide treatment did not have a statistically significant effect on the viability of the cells (IC_0 value). 200 µM of temozolomide treatment decreases the cell viability by 80% (IC_{20} value). The temozolomide concentration, which reduced the number of viable cells by 50%, was determined as 750 µM (IC_{50} value). In the experiments, the final DMSO concentration did not exceed 0.75% and these concentrations were determined not to affect cell viability (Figure 3.3).

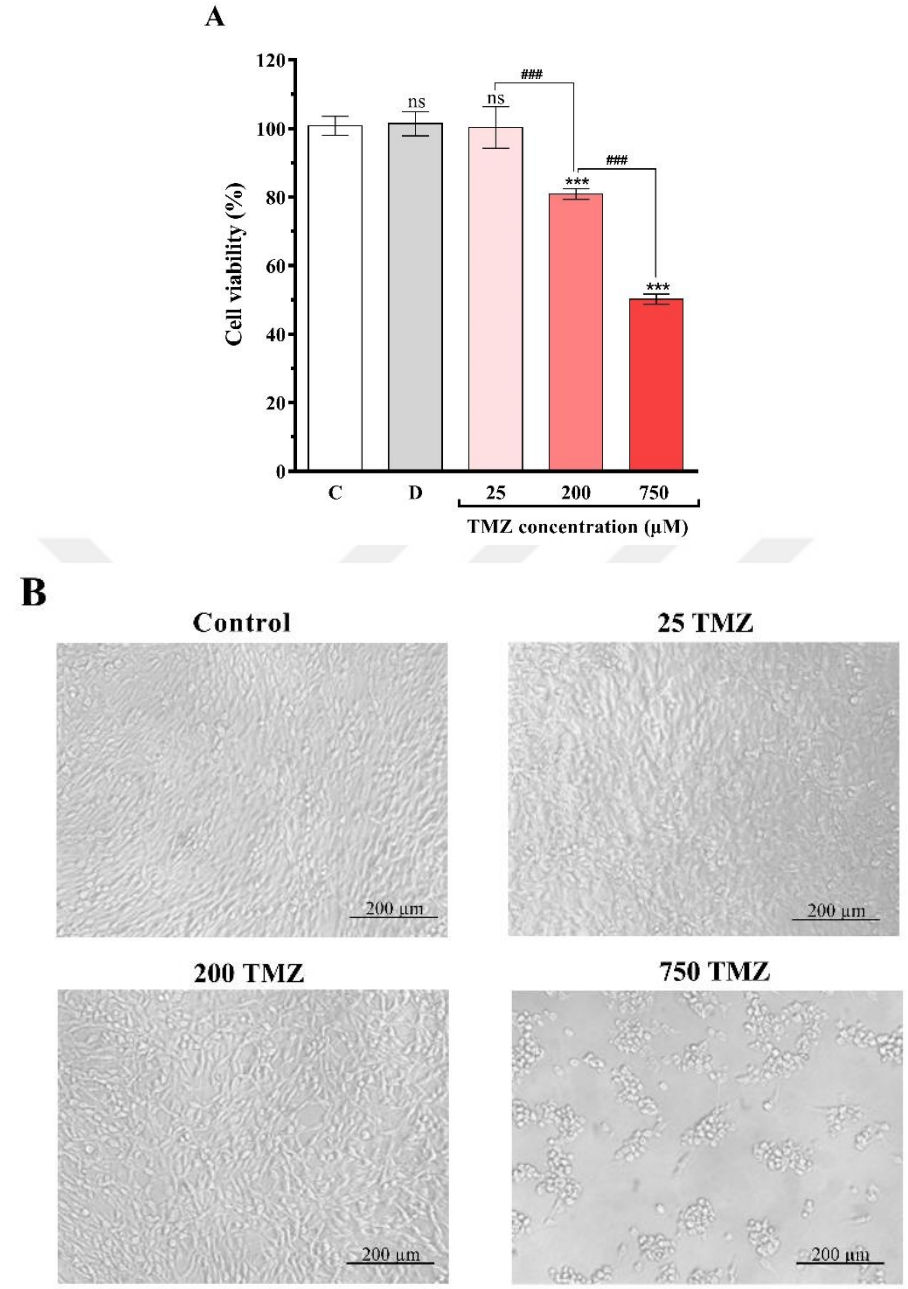

Figure 3.3: Temozolomide treatments for U87MG viability for 48h. **A)** Cell viability was determined by MTT assay. The untreated cells (C, control), treated with 0.75% DMSO (D) **B)** Image of cells after TMZ treatments. Vertical bars indicate standard deviation values. ***P<0.001 versus control (C) group, $^{###}P$<0.001 indicates statistical difference between different groups. *C* control, *D* DMSO and *ns* not significant.

3.3. PROTEIN CONCENTRATIONS OF CELLULAR EXTRACTS

Protein concentrations of the cell extract obtained in the context of the thesis were determined by a commercial kit (SMART^{TM} BCA Protein Assay Kit, iNtRON Biotechnology) according to the BCA method.

The bovine serum albumin (BSA) standard in the kit was diluted with dH_2O to the concentrations of 25, 125, 250, 500, 1000, 1500 and 2000 µg/mL and a standard graph was made according to BCA method (Figure 3.4). Protein concentrations of all samples were determined using the equation from the standard graph. The protein concentrations of the cellular extracts obtained from the control and experimental groups were found in the range 9000 – 20000 µg/mL. Exosomal protein concentration in both control and experimental group were found in range 3000 – 9000 µg/mL.

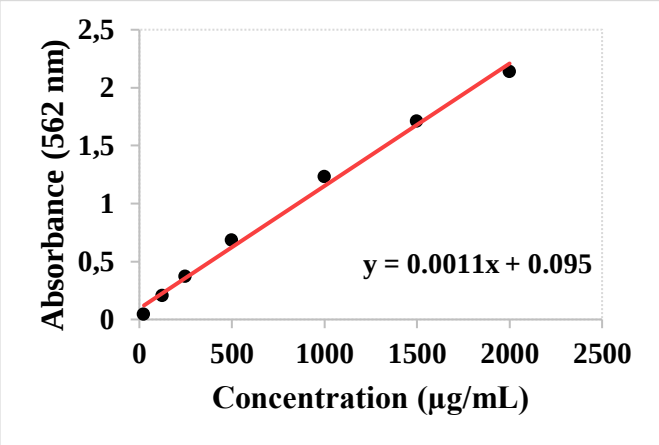

Figure 3.4: Protein standard graph made by using Bovine Serum Albumin (BSA) standards (R^2=0.994).

3.4. ELECTROPHORETIC ANALYSIS

All of the protein extracts used in the thesis were mixed with sample loading buffer and loaded onto the gel with 30 µg of protein in each well. After the proteins were separated at 200V constant voltage, Coomassie Blue staining method was applied to make the proteins visible on the gel. The bands were visualized on the ChemiDoc MP (Bio-Rad) imaging system using ImageLab 5.2.1 software. An example of gel image is given in Figure 3.5.

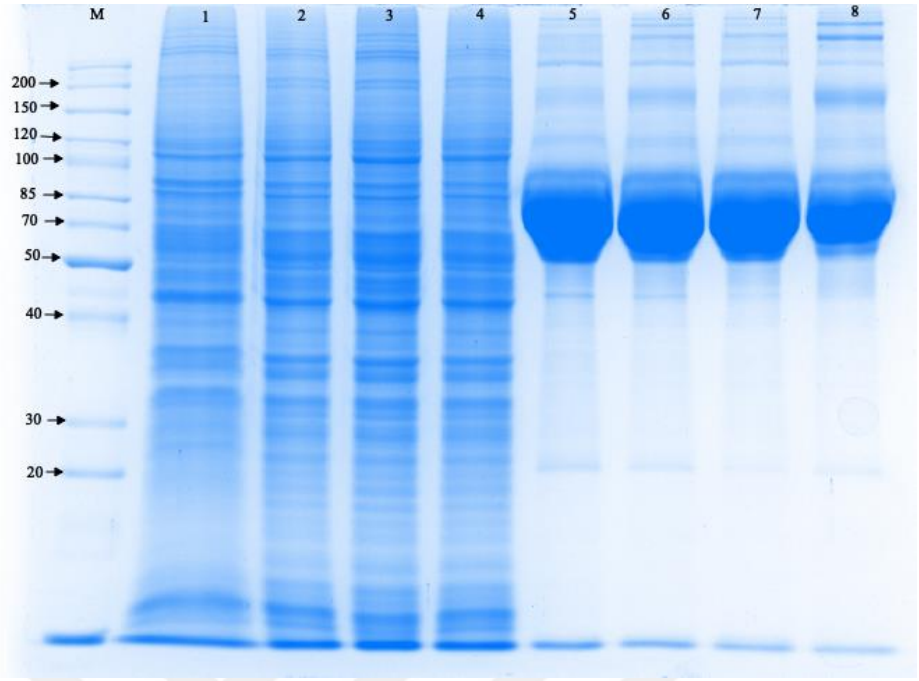

Figure 3.5: Sample gel image stained by Coomassie staining method.

M: Marker (Pierce® Blue Prestained Protein MW Marker Mix, Thermo Scientific 266121), protein extract of control group U87MG glioma cells with no TMZ treatment, 2: 25 μM TMZ treatment, 3: 200 μM TMZ treatment, 4: 750 μM TMZ treatment, 5: exosomal fractions of control group U87MG glioma cells with no TMZ treatment, 6: 25 μM TMZ treatment, 7: 200 μM TMZ treatment, 8: 750 μM TMZ treatment.

3.5. CHARACTERIZATION OF EXOSOMES

Exosome isolation was performed as described in section 2.8 and proteins that are extracted from exosomes. Equal amount (30 μg) of Alix, CD63, CD81, TSG 101 as exosomal marker proteins and Hsp70 separated by SDS-PAGE (as described in Section 2.11) and visualized by western blot analysis. Figure 3.6A shows the expression of these markers in exosomes isolated from U87MG cells compared to equal amount of the U87MG cell lysate. CD63 and CD81 are absent in lysate but found in abundance in the exosomal preparation. It could be possible to conclude that the U87MG cells secrete exosomal vesicles into their medium. Figure 3.6B indicates the characterization of exosomes by Scanning Electron Microscope (SEM). Exosomes had a spherical shape with a diameter of about 50-85 nm was shown in Figure 3.6B.

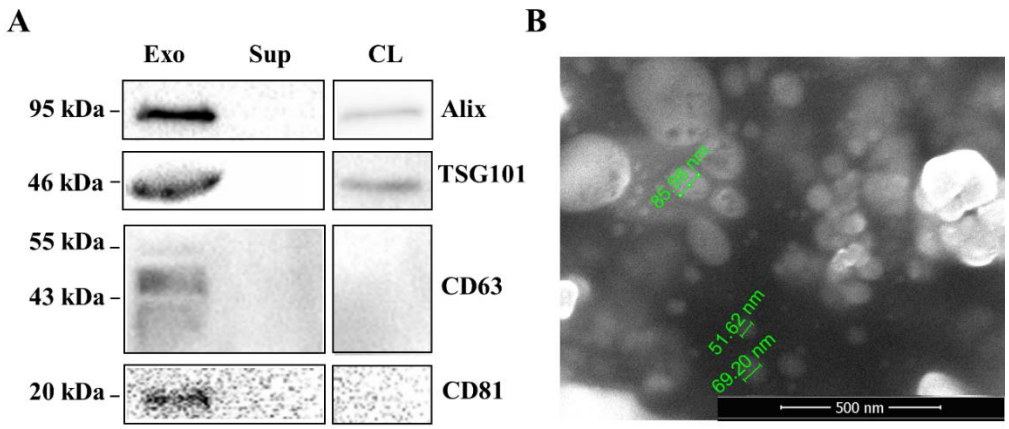

Figure 3.6: Characterization of exosomes released from U87MG by western blotting and SEM imaging. **A)** Western Blot characterization of glioblastoma exosomes. **B)** The characterization of exosome was performed using Scanning electron microscope (SEM).

Exosomes (Exo), Supernatant (Sup), Cell Lysate (CL) from glioblastoma cells were separated on SDS–PAGE and electroblotted PVDF membrane which was incubated with Alix, TSG 101, CD63 and CD81 are known exosomal markers. Molecular weight marker is shown at left. Exosomes from U87MG cells are imaged by SEM (scale bar 500 nm).

3.6. IMMUNOLOGICAL ANALYSIS

Proteins that are extracted from exosomes separated according to their molecular weight in SDS-PAGE were transferred to PVDF membrane using Wet / Tank Blotting System (Bio-Rad) (as described in Section 2.13) and incubated with protein-specific antibodies (see Table 2.6). In order to detect target protein bands; membranes were incubated with primary antibodies and HRP- conjugated secondary antibody, respectively. In the aim of normalizing the membranes (for cell lysate samples), analyzed targeted bands, the membranes were stripped from the antibodies and reconstituted for immunological analysis. After blocking the membranes, they were incubated with GAPDH antibody then HRP-conjugated secondary antibody. Chemiluminescence on the membrane was visualized by using the imaging kit (Super Signal ™ West Pico PLUS Chemiluminescent Substrate, Thermo Scientific) and analyzed using the ImageLab 5.2.1 software on the ChemiDoc MP (Bio-Rad) imaging device. Normalization calculations were performed using ImageLab 5.2.1 software. In the thesis, all immunological analysis was performed at least 3 replicates with different experimental sets. In the evaluations, the expression of the analyzed proteins was accepted as 100% in the control group which was treated with temozolomide and the results were compared with the experimental groups. The consistency between the replications in the experimental groups was determined using the one-way ANOVA test and the Tukey test as the post hoc test.

Depending on the treatment with the various concentrations, differences in intracellular and extracellular Hsp70 levels which by determined with western blot analysis were observed (Figure 3.7).

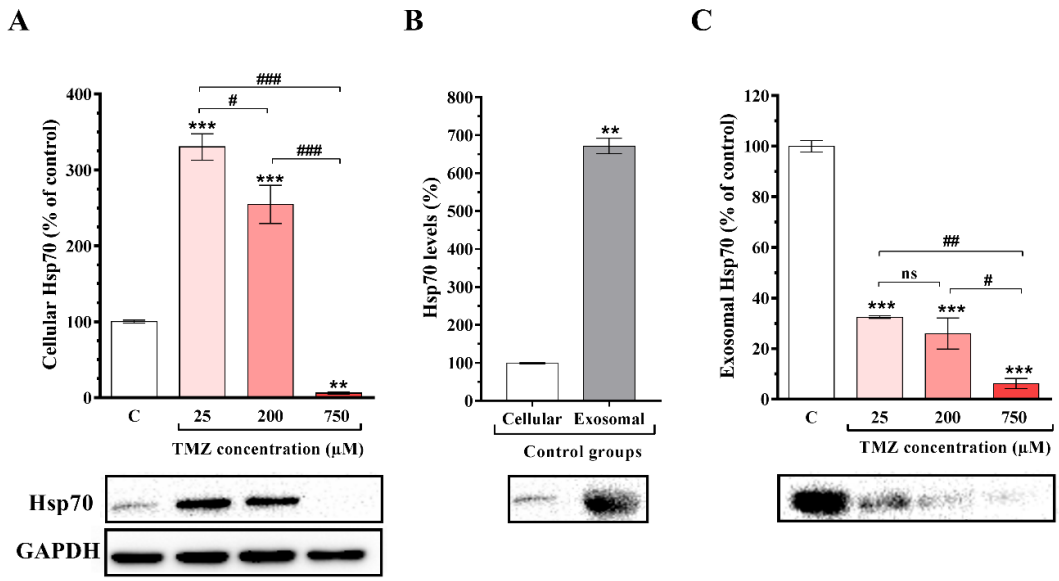

Figure 3.7: The effects of temozolomide treatment on expression of Hsp70. For **A** and **C)** *P* values were determined using one-way ANOVA followed by Tukey's post hoc test. ***P<0.001 versus control (C) group. $^{\#}P$<0.05 and $^{\#\#\#}P$<0.001 indicate statistical difference between different groups For **B)** **P < 0.01, P value was determined by Student's t-test. Vertical bars indicate standard deviation values.

The results showed that exosomal Hsp70 expression levels reduced by TMZ treatments whereas cellular Hsp70 expression increased in a dose-dependent manner except 750µM. When the intracellular and exosomal Hsp70 levels of the control group were compared, it was determined that the exosome had 6 times higher Hsp70 levels. TMZ concentrations of 25 and 200 µM respectively (0% and 20% toxic doses respectively) resulted in a 230% and 154% increase in Hsp70 in the cell, resulting in a 68% and 74% reduction in exosomes. This result shows that the content of exosomes secreted from U87MG cells was decreased in terms of Hsp70 as a result of TMZ treatment.

3.7. CELL MIGRATION ASSAY (*in vitro scratch assay)* ANALYSIS

After the treatment of temozolomide in the concentration range between 25 and 750 µM, the cell proliferation graph of the U87MG cells for 0, 24, 48 hours, the percentages of the cell migration are given in Figure 3.8.

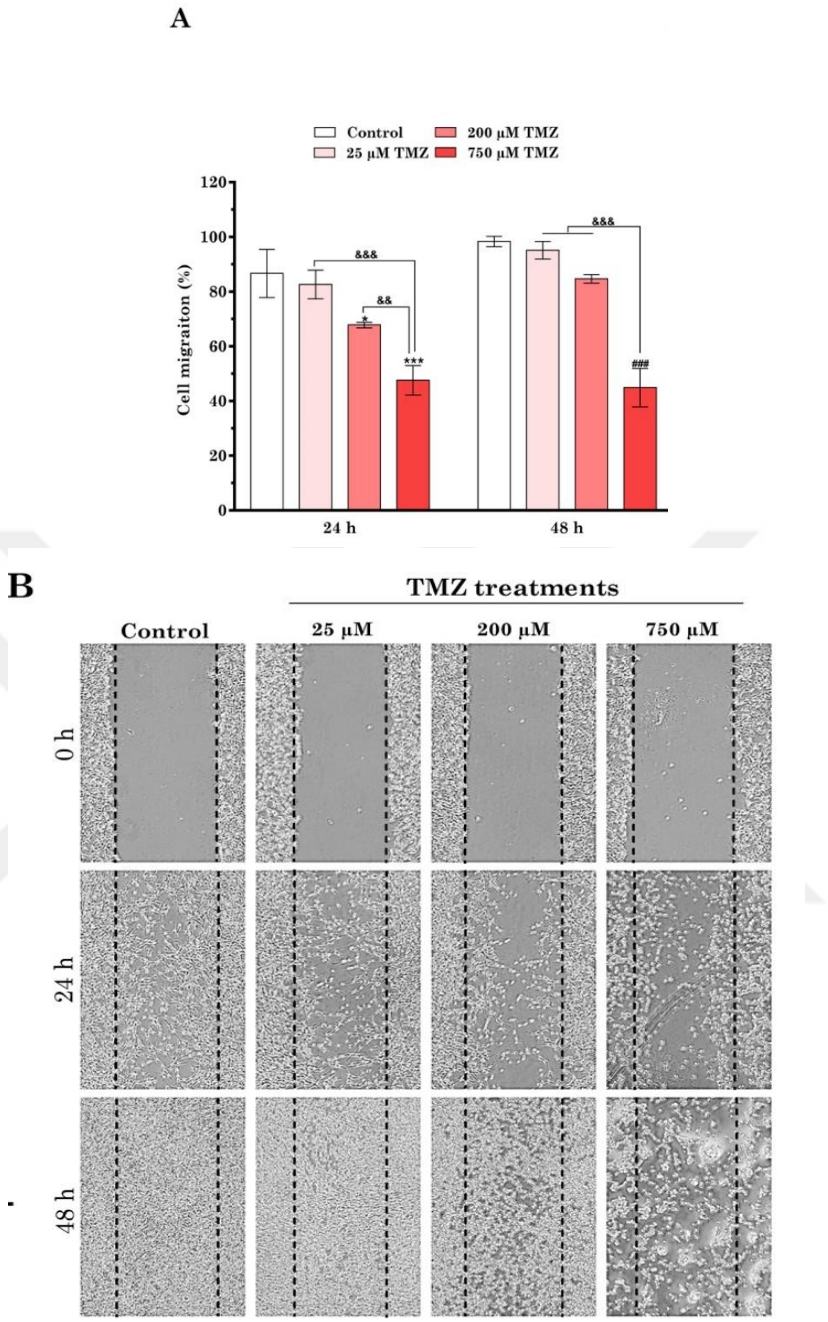

Figure 3.8: The effect of temozolomide on the cell migration. **A)** Cell migration (%) was determined by *in vitro* scratch assay. Vertical bars indicate standard deviation values. **P*<0.05 and ****P*<0.001 versus control group for 24h, $^{\#\#\#}P$<0.001 versus control group for 48h, $^{\&\&}P$ <0.01 and $^{\&\&\&}P$ <0.001 indicates statistical difference between different groups **B)** Images of wounded monolayer of U87MG taken at times 0, 24 and 48 h after treatment with TMZ.

For 24- and 48-hour incubation time, a statistically significant difference was observed in the percentage of cell migration between 200 and 750 µM, and 25 and 750µM temozolomide concentration in U87MG cells compared to control (P <0.001). On the other hand, the graph

demonstrated that there were no significant differences between control and 25μM, and 25μM and 200μM concentrations. The percentage of migration of the control was 86.7% after 24 hours of incubation, 82.6% for 25 μM, 67.8% for 200 μM and 47.6% for 750 μM. After 48 hours of incubation, the control had a 98.3% migration rate, 95.1% for 25 μM, 84.6% for 200 μM and 44.8% for 750 μM.

Temozolomide has a significant effect on cell migration in a dose-dependent manner. According to the obtained data, it is seen that cell migration slows and decreases as the concentration of temozolomide increases.

4. DISCUSSION

Cancer is at the top of the list of diseases that cause death in the world [1]. Malignant gliomas constitute the most common primary brain tumors in adults and rank among the most devastating and aggressive types of human cancer due to their dismal prognosis. The median survival rate for GBM patients is only 8-12 months from diagnosis [4]. The reasons for the poor prognosis related to GBM are: (1) Despite the rare systematic metastasis, tumor cells leak the surrounding brain tissue because surgical resection is limited. (2) Also, the role of the BBB as an obstacle to appropriate chemotherapy drugs for brain tumors. Only small molecules are allowed to penetrate the BBB to reach their target. (3) GBM is resistant to many cytotoxic drugs. MGMT expression is considered the main resistant mechanism to DNA alkylating agents such as temozolomide.

Temozolomide (TMZ), a DNA alkylating and an oral chemotherapeutic drug, is used as first line treatment in glioblastoma treatment for a long time [113]. TMZ is the most promising therapeutically in glioma treatment. Due to its excellent ability to penetrate into all body tissues including the BBB and with this feature distinguishes it from the other chemotherapeutic drugs. It is administrated orally and absorbed rapidly [43]. TMZ targets GBM at a molecular level by breaking DNA double strand and reduce the activity of MGMT, which is a DNA repair enzyme promotes tumor cell death. It is known that brain tumor cells express low levels of this enzyme [114].

It is now well understood that extracellular vesicles (EVs), a huge family with different sizes and are considered being important elements in the cell-to-cell communication beside hormones, cytokines or neurotransmitters [12]. EVs are broadly classified according to their biogenesis mechanism, e.g., budding from the surface of cells (microvesicles and large oncosomes) or forming within endosomal multivesicular bodies (MVBs; exosomes) [115]. All of these vesicles to involved in various biological process, particularly cellular communication by a serious impact on both the local environment and distal tissues [13].

Particularly exosomes, the smallest vesicle of EVs as range 30 to 150 nm in size, spherical structures surrounded by a lipid bilayer [14]. They contain transferrin receptor, tetraspanins (CD9, CD63, CD81, and CD82), and glycosylphosphatidylinositol (GPI)-anchored membrane proteins [116]. Almost all kinds of cells in the human body are able to release such vesicles to

the extracellular environment. These tiny vesicles involve many different diseases like neurodegenerative disorders, cancer, and AIDS [15]. Numerously studies demonstrated that exosomes released soluble factors to cross talk between cells, thus they can convey to communicate between cancer and healthy cells [117]. Thus, cancer cells by virtue of exosomes can escape the immune system, inhibit immune cells, increase angiogenesis around the tumor microenvironment, and acquire drug resistance, invasion, and metastasis capabilities. Exosomes allow tumor progression and metastasis via oncogenic signal proteins, ligands, enzymes and miRNAs that they carry on their surface or inside the vesicle.

Stress proteins or Hsps constitute a large family of proteins with conserved structures. Their expression levels increase in various types of cancer, and overexpression of Hsps suggests a poor prognosis in some types of cancer including gliomas and in response to treatment [20].

Various studies have shown that overexpression of Hsps is associated with differentiation, invasion, metastasis, and proliferation of tumor cells, drug resistance and cell survival under lethal conditions, making these proteins a good target in cancer treatment. Inhibition of Hsp expression, which is particularly effective in inhibiting cell apoptosis and increasing resistance to chemotherapeutic drugs, is being used as a new approach in the treatment of cancerous disease with an increasing number of cases [118].

Although these proteins have traditionally been regarded as intracellular molecules, recent research has shown that a new secretion pathway with exosomes has emerged for these proteins, and thus they can be seen in extracellular localizations or in the blood [22]. In a study conducted [23] on brain tumor cell lines, exosomes were identified, their Hsp contents as potential sources of extracellular Hsps were characterized. Hsp27, aB-crystallin, Hsp60, Hsp70, Hsc70 and Hsp90 have been shown to be present in GBM released exosomes [24]. Hsp contents of exosomes and their roles in tumor development are of great interest among researchers. Further studies are needed to explain the localization of uncommon exosome membrane stress proteins and Hsp contents of exosomes. Following the suggestion of a special relationship between stress proteins known to be associated with various diseases and brain tumors [119], a number of studies have been conducted on this subject. In particular, studies have shown that increased expression of Hsp70s is found in many brain tumors [120].

It has been reported that Hsp70 expression is higher in non-malignant brain tissues in GBM and the expression level of this protein is related to the degree of malignancy of the glial tumor. Elevated levels of Hsp70 play a key role in the regulation of malignancy, cell proliferation, resistance to therapies and poor prognosis [121]. Hsp70 was previously described in the intracellular localization of tumor cells but was not only found on the membrane of tumor cells [122]. However, four different forms are known today for Hsp70: (1) intracellular Hsp70, (2) extracellular Hsp70, (3) membrane Hsp70 and (4) exosomal Hsp70. Intracellular Hsp70 has important roles in crucial steps of tumorigenesis such as angiogenesis, invasion, and metastasis [123]. Tumor-derived extracellular Hsp70 is secreted by necrotic tumor cells, could enhance tumorigenesis through entering adjacent cells or blood circulation [124]. The secretory mechanisms of extracellular Hsp70 are complex and incompletely understood. Hsp70 is also actively secreted by the tumor cells via exosome pathways. Exosomal Hsp70 can induce the metastasis and cytolytic activity of tumor cells [89]. So far, clear functions of membrane Hsp70 remains unknown.

In this thesis, we aimed to investigate the effect of temozolomide (TMZ) on the Hsp70 expression levels in both intracellular and exosomes of U87MG human glioblastoma cells.

In this context, the effects of TMZ on cell viability were determined by MTT analysis. IC_{50} value (half-maximal inhibitory concentration) of TMZ was determined as 750 μM. In addition, when the toxic effects of DMSO in which TMZ was dissolved, the highest concentration (0.75%) treated in the culture medium did not have a statistically significant effect on the viability of the cells. The concentrations of TMZ to be applied to glioma cells (see Table 2.1) were determined according to the IC_{50} graph obtained from MTT analyzes performed. Three different concentrations of TMZ were administered to U87MG cells, with doses of these agents that did not affect cell viability in any way (no cytotoxic or proliferative effect), affecting only 20% of viability and affecting viability at 50% (see in Figure 3.2). The western blotting technique was used to determine the effects of TMZ on the expression of Hsps of cellular and exosomal origin in glioma cells.

Our thesis demonstrated that the U87MG cell line releases exosomes into its conditioned medium. Exosomes were isolated then, confirmed by scanning electron microscopy (SEM) analysis, illustrating cup-shaped and range in size between 30–100 nm in size, and the

expression of the most common exosomal markers Alix, Tsg101, CD63 and CD81 by Westen Blot analysis.

In the second stage of the thesis, the effects of TMZ applied to cells on expression of Hsp70 were evaluated. Depending on the TMZ concentration treated, differences in intracellular and extracellular Hsp70 levels, which by determined with western blot analysis were observed. We showed that exosomal Hsp 70 expression levels reduced by TMZ treatments whereas cellular Hsp70 expression increased in a dose-dependent manner. When the intracellular and exosomal Hsp70 levels of the control group were compared, it was determined that the exosome had 6 times higher Hsp70 levels except for the highest dose of TMZ.

TMZ concentrations of 25 and 200 μM respectively (0% and 20% toxic doses respectively) resulted in a 230% and 154% increase in Hsp70 in the cell, resulting in a 68% and 74% reduction in exosomes. This result shows that the content of exosomes secreted from U87MG cells was decreased in terms of Hsp70 as a result of TMZ treatment. Hsp70 decreased in exosome content was also found to play an important role in decreasing cell migration (the result is given below). The 750 μM TMZ resulted in decreased Hsp70 levels both in the cell and in the exosome. This may be related to the release of Hsp70 as extracellular in the cells because of high cell death rates due to the toxicity of 750 μM TMZ.

Cancer invasion is complex and involves integrated biochemical processes requiring coordinated efforts of intracellular and extracellular interactions [125]. Therefore, cell surface adhesion molecules play important roles in the cell to cell or cell to the microenvironment.

Cell migration and metastasis are considered as a key event in the glioma progression. It is also indicated in many previous studies that exosomes modulate the tumor microenvironment by promoting metastasis and tumor growth. Exosomes actively participate in the metastasis process in different ways. Particularly, GBM exosomes have mRNA and proteins associated with metastasis and promote endothelial cell proliferation. This information was also supported with further studies in U87MG cells derived exosomes exhibited a strong migration ability [126]. Moreover, there are several studies that may confirm our results. Recent studies reported that exosomes released from fibrosarcoma and malignant melanoma cells carry MT1-MMP (Membrane-type matrix metalloproteinase). It is known that MT1-MMP activates pro-MMP-2 in the extracellular matrix causing type 1 collagen and gelatin destruction and contributes to

invasion [127]. In addition, exosomes attained from glioblastoma and breast cancer cells increase cell mobility by activating extracellular vesicles by Hsp90alpha [128].

In this thesis, we investigated the effect of different concentrations of TMZ on the cell migration and motility of U87MG cells by *in vitro* scratch assay. With comparable initial scratch sizes, cells showed the limited growth under TMZ demonstrated significantly decreased cell migration. This migration assay revealed that TMZ has an effect on slowing cell migration in a dose-dependent manner. The 750 μM of TMZ (IC_{50} concentration) showed the highest impact on the cell migration but it also reduced the cell viability. The 200 μM of TMZ (IC_{20} concentration) also slowed the scratch closure with moderate consequences in the cell viability. In addition, there was no statistically significant effect of 25 μM TMZ (IC_0 concentration) on cell migration compared to control. Tumor migration ability is an important biological characteristic of malignant tumors and inhibition of glioma cell migration is critical to the treatment of malignant gliomas. However, the study of the migration ability of temozolomide to glioma is not sufficient. Nevertheless, the effect of TMZ on the inhibition of cell growth and migration both U251 and U87MG cells have been showed in several studies [129].

Our western blot results showing decreased Hsp70 level in exosomes due to TMZ treatment here, we proposed that our migration analysis findings point out that the content of exosomes released by U87MG cells is important in stimulating cell migration and TMZ have an inhibitory effect on migration. Our findings also are supported with a previous study, which temozolomide reduced cell viability, caused cell cycle arrest in the gap 2/mitosis phase, inhibited cell migration and promoted autophagy in U251 cells thus reduced their viability and migration [130].

A lot of literature information mentioned above supports our study. In brief: 1) Hsps play a key role in the tumorigenesis process including angiogenesis, invasion, proliferation, and migration [21]. 2) Elevated cytosolic Hsp70 levels have been found in almost all tumors and its expression is related to tumor progression, resistance to therapy and formation of metastasis. 3) Hsp70 proteins are highly expressed in glioma cell lines [131]. 4) TMZ is known as an inhibitory anticancer drug on the development of cancer cells, reducing cell growth and spreading to distant parts of the body [8].

In the light of those results, all the studies performed to reduce the extracellular -nonclassical pathway expression level of stress proteins in glioma cells can help to develop new therapeutic approaches for the treatment of brain tumors with high mortality rates.

5. CONCLUSION AND RECOMMENDATIONS

In this thesis, exosomes were characterized by Western Blots and SEM. Then, the effects of temozolomide on the expression of Hsps in exosomes released from U87MG cells were analyzed. Our data nonetheless show initial evidence that temozolomide has the elevating effect on the expression level of Hsp70 intracellular, whereas the extracellular-nonclassical pathway expression level of Hsp70 reduced in glioma cells. These findings may help to develop chemotherapeutic agents and related therapies for the treatment of brain tumors with high fatal consequences.

The results are considered as a promising approach in slowing cell migration and as a conclusion slowing metastasis by decreasing the amount of the Hsp70 by TMZ treatment at the highest dose. In order to confirm the findings, further research at molecular levels is required. The isolation procedure also will be enhanced to obtain more exosomes. Further analysis will be required as to determine exosome releasing from donor cells and uptaking by recipient cell via Nanoparticle Tracking Analysis (NTA). In addition, it is necessary to determine whether the source of Hsp70 is membrane bound-Hsp70 or intracellular Hsp70.Then membrane bound Hsp70 expression level should be determined. On this purpose, it is needed to be analyzed by further experiments.

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