THE ROLE OF C60 NANOPARTICLE ON APOPTOSIS AND AUTOPHAGY IN HUMAN GLIOMA CELL LINE

Master Thesis

Aryan Mahmood FARAJ

Biology Department

Ass. Prof. Dr. Can Ali AĞCA

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REPUBLIC OF TURKEY
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This master thesis on 17.01.2017 by the following jury memebers (The appropriate one will be stay, the other will be deleted) **Unanimously has been accepted.**

Prof. Dr.	Ass. Prof. Dr.	Assoc. Prof. Dr.
Victor NEDZVETSKI	**Abdullah ASLAN**	**Can Ali AĞCA**
President of jury	**Member**	**Member**

The above results has been approved by

Prof. Dr. İbrahim Y. ERDOĞAN

Director of the Institut

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ÖZET

Glioblastoma, kemoterapi ve radyoterapiye karşı yüksek direnç gösterebilen en agresif tipteki astrositom türüdür. Nanopartikül C60 fulleren, güçlü antioksidan ve antikanser aktivitesi göstermektedir. Birçok kemoterapi maddesinin aksine, C60 fulleren yüksek konsantrasyonlarda dahi toksik etki göstermemektedir. Pristine C60 fullerene bir çok biyomedikal uygulama için umut verici bir aday olarak görülmektedir. C60 fulleren'in kanser üzerindeki etkisi kapsamlı bir şekilde incelenmiş olmasına rağmen, glioblastomada, otofaji ve apoptozis arasındaki ilişkideki olası rolü henüz ele alınmamıştır. Glioma hücre hatlarında, otofaji ve apoptoz arasındaki ilişki mekanizması hala tam olarak bilinmemektedir. Bu nedenle, insan glioblastoma astrocytoma hücresi üzerinde C60 fulleren'nin p53/Kaspaz-9/Kaspaz-3/p21/PARP ve otofaji yolağı üzerindeki etkisi araştırılmıştır. Glioblastoma U-373 hücreleri, 24 saat süreyle farklı konsantrasyonlarda (0,5, 1 ve 2 μM) C60 fulleren ile tedavi edilmiştir. Hücre proliferasyonu ve canlılığı MTT testi ile belirlenerek, ROS seviyeleri sırasıyla ROS/RNS ve DCFD testi ile ölçülmüştür. p53, Kaspase-9, Kaspase-3, p21, PARP Beclin1 ve LC3 proteinlerinin düzeyleri western blot ile belirlenmiştir. MTT analizinin sonuçları, C60 fullerenin hücre proliferasyonunu ve hücre canlılığını önemli ölçüde azalttığını göstermiştir. Tüm dozlar, C60 fulleren ile tedavi edilen U373 hücre hattında ROS ve NO düzeylerini arttırmıştır. Beclin-1'in düzeyi ve LC3-II / LC3-I oranı, C60 fulleren tedavisi ile önemli ölçüde indüklenmiştir. Apoptozisin, regülasyonunun önemli ölçüde artırıldığı gösterilmiştir. C60 fullerene tedavisinin U373 hücre hattında belirgin şekilde ve kaspaz-9/3 ve p53'ün aktivitesini artırdığı ve apoptozisi uyardığı belirlenmiştir. 24 saatlik tedavi süresinin ardından, malign glioma U-373 hücre hattında C60 fulleren'nin p53 bağımlı p21 proteinin ve PARP upregülasyonu sağladığı ve böylece hücre döngüsünün durmasını tetiklediği bulunmuştur. C60 fulleren, doz bağımlı olarak U373 hücrelerinde proliferasyonu yavaşlatmış, apoptozis ve otofajiyi indüklemiştir. Genel olarak bulgularımız suda çözünür C60 fullerenin, p21/PARP,Beclin1/LC3 ve p53/kaspaz-9/kaspaz-3 sinyal yolaklarını indükleyerek U373 hücrelerinde hücre döngüsünün düzenlenmesini, apoptozis ve otofajiyi indüklediğini ve C60 fullerenin glioma kanseri hücrelerindeki etkisine ışık tuttuğunu önermektedir.

Anahtar kelimeler: C_{60} fullerene, glioma U373, apoptozis ve otofaji.

THE ROLE OF C_{60} NANOPARTICLE ON APOPTOSIS AND AUTOPHAGY IN HUMAN GLIOMA CELL LINE

ABSTRACT

Glioblastoma is one the most aggressive types of astrocytoma, resistant chemo- and radio-therapy. Nanoparticles C_{60}fullerene are the power antioxidant and demonstrate anticancer activity. In contrast to many chemotherapy agents, this fullerene absolutely non-toxic in wide range of concentrations. Pristine C_{60} fullerene is a promising candidate for many biomedical applications. Despite the fact that, the effect of C60 fullerene in cancer has been extensively studied, the potential regulation of between autophagy and apoptosis has not been addressed in glioblastoma. Molecular mechanism of crosstalk between autophagy and apoptosis in glioma cell line remain unknown. Therefore, we investigated the effect of C60 fullerene on p53/caspase-9/caspase-3/PARP and autophagy pathway on human glioblastoma astrocytoma cell. Glioblastoma U-373 cells were treated with different concentrations (0.5, 1 and 2 μM) C_{60} fullerene for 24 hours. Cell proliferation and viability were detected by MTT assay. ROS levels were measured by ROS/RNS and DCFD assay respectively. Changes of p53, Caspase-9, Caspase-3, PARP, p21, Beclin1 and LC3protein expressions were analysed by western blotting .Results of MTT assay showed that C60 fullerene significantly decreased cell proliferation and cell viability. All doses C_{60} fullerene treatment increased ROS and NO levels in U373 cell line. The expression of Beclin-1 and ratio of LC3-II/LC3-I was significantly induced by treatment of C60 fullerene. We showed that apoptosis was significantly upregulated in U373 stimulated with C_{60} fullerene induced apoptosis and increased the activity of caspase-9/3 and p53. We found that C_{60} fullerene induced cell cycle arrest, p53-dependent upregulation of p21 and activated PARP in human malignant glioma U-373 cell line after 24-hour treatment. C_{60} fullerene inhibited the proliferation and induced apoptosis and autophagy in U373 cells in a dose-dependent manner. Overall, our findings have suggested that water-soluble C_{60} fullerene induces cell cycle arrest, apoptosis, and autophagy in U373 cells through inducing p21/PARP, Beclin1/LC3 and activating p53/caspase-9/caspase-3 pathway signaling pathways. Our findings shed light on the effect of C60 fullerene in glioma cancer cells.

Key words: C_{60} fullerene, glioma U-373, apoptosis and autophagy.

1. INTRODUCTION

1.1. Overview

Cancer is a genetic disease, characterised by stop obeying of normal cells and outgrowth of unbalance version, grow continuously without control and divers throughout the body (Khan and Pelengaris 2013). Referred to Hallmark of cancer, cancers or every cancer cells should have these characteristics, proliferation independent to exogenous mitogens, unaffected by growth inhibitory signals, resistance to apoptosis, immortality, angiogenesis and ability to invade surrounding tissue (Hanhan and Weinberg 2000). Primary brain tumour characterised by arising from brain cells, such as glioma arises from the glial cell. Whereas, secondary brain cancer comes and spread from another cancer from other parts of the body and colonises in the brain (Friedman and Liau 2013). Based on the growth factor signalling pathway, cancer cells use three main cellular strategies such as changes in extracellular growth signals, changes in trans-cellular signals and signalling messengers. However, many cancer cells depend on autocrine stimulation instead of growth factors, overexpression of growth factor receptors, induce the intracellular receptors, and depend on them rather than extracellular growth receptors (Hejmadi 2010). Among the other brain cancers, glioma is arising from glial cells and one the most common brain tumour, most glioma arises from astrocytes or oligodendrocytes (Friedman and Liau 2013). 32% of central nervous system tumours and 80% of malignant central nervous system tumours belong to glioma (Agnihotri et al. 2013). The most aggressive glioma is glioblastoma belong to grade IV glioma, characterised by fast-growing and rapidly invade to the adjacent tissue (Friedman and Liau 2013). Glioma and glioblastoma most common framework of primary brain tumours, in 1863 Dr Rudolf Virchow has first identified glioblastoma, 54% of all astrocytic tumours are glioblastoma and more common in men than a woman by ratio (1.58:1) and twice in Caucasian rather than African-American (Agnihotri et al. 2013).

Nanotechnology focuses on the nano-scale materials (Stern et al. 2012). Nanomaterials sizes arranged between 1 and 100 nanometres in one or more dimension (Ying et al. 2013). The carbon nano-materials are hollow, circle and more precise atomic composition (Baldrighi et al. 2016). Nano-materials synthesised from organic and inorganic materials with the aim of enhancing the act of medicines, therapeutic efficiency and reduction the systemic side effect (Panzarini et al. 2013). Because of physiochemical properties, nanomaterials are more common in research and industry, like ultra small size, increased a proportion of surface area to volume modification and good biocompatibility (Ying et al. 2013). Carbon nanoparticles show more diversity in morphology, physical and chemical properties or reactivity. The physical properties make carbon nanoparticle different interaction with cells and tissue, chemical properties by covalent and non-covalent functionalization, allow them to change their surface charge, introduce fluorescent targeting molecules, cell and diseases specific targeting molecules, magnetic and contrast agent (Baldrighi et al. 2016).

Apoptosis is the well-known programmed cell death, has specific morphologic and biochemical character of dying cells. The characters of apoptosis is counting as cell shrinkage, fragmentation of nucleus and condensation of chromatin, membrane blebbing (Su et al. 2015 and Nishida et al. 2008), chromosomal DNA fragmentation (Su et al. 2015) and defeat of adhesion to adjacent or extracellular matrix (Nishida et al. 2008). The biochemical changes in apoptosis cell dying comprise chromosome cleavage, phosphatidylserine exteriorization and cleave a number of intracellular substrates by precise proteolysis (Ouyang et al. 2012). If, DNA damage is unable to fix, subsequently apoptosis will be occur. The apoptosis induces by the present of the two pathways, intrinsic (mitochondrial) and extrinsic (death receptor) pathway (Eum and Lee 2011). The extrinsic pathway triggered by plasma membrane death receptors, Fas and other comparable receptors for example tumour necrosis receptor-1 and its family, binding to their extracellular ligands such as Fas-L. The death stimuli occur when Fas-L joins with Fas to make a death complex activates pro-caspase-8, which trigger to the second protein of apoptosis process pro-caspase-3 (Kerr et al. 1972). Intracellular stimuli, deprivation of growth factor, DNA damage and oxidative stress are induced apoptosis pathway intrinsically by the configuration of a multifaceted called the apoptosome which composed of caspase-9, apoptotic protease activating factor (Apaf-1) and cytochrome-c

(Su et al. 2015). Beneath the director of mitochondrial pro-enzymes, the intrinsic pathway guides to apoptosis. If the extracellular and intracellular signals induced apoptosis, the mitochondrial outer membrane become permeable to cytochrome-c, then released to the cytoplasm and recruits Apaf-1 and caspase-9 to create apoptosome. Any abnormal expression of some key regulator of apoptosis may lead to cancer (Kerr et al. 1972). The success malignant cells metastasis is an ability to apoptosis-resistant, it isn't needed for all steps of metastasis but more necessary during the loss of cell-cell and cell-extracellular matrix (ECM) contacts because when the cell detached from the ECM, induce anoikois apoptosis cell death (Su et al. 2015). The caspase-independent cell death programmed necrosis described as a backup mechanism for apoptosis when caspases are inactivated, however, necroptosis invert to apoptosis in the presence of a specific necroptosis inhibitor such as necrostatin-1 (Nec-1) (Ouyang et al. 2012).

The second type of programmed cell death autophagy (Ouyang et al. 2012) is a cellular strategy for survival under stress or unwanted condition (Su et al. 2015), defined by Christian De Duve in 1963 (Stern et al. 2012). Autophagy described as a cellular self-scarifying mechanism (Ghavami et al 2014) and the catabolic process beginning with autophagosome double membrane-bound structure (Ouyang et al. 2012). Autophagy involves fusion of autophagosome of cellular proteins or pathogens in double membrane structures with the lysosome to forming autolysosomes (Stern et al. 2012). Autophagosome is surrounding and package the cytoplasmic macromolecules, organelles (Ouyang et al. 2012) and proteins, that delivery to lysosome and vacuole for digesting and recycling, which has an important role in the cell fates (Su et al. 2015 and Ghavami et al. 2014)

Autophagy is macroautophagy, microautophagy and chaperon-mediated autophagy (CMA), induced by various forms such as starvation, oxidative stress and mitochondrial effect etc (Su et al. 2015). Autophagy process included nucleation, elongation, autophagosome and autolysosome formation, triggered by nutrient deprivation, hypoxia, ROS, drug stimuli, and endoplasmic reticulum (ER) stresses (Ghavami et al 2014). The pathway of autophagy-related to some autophagy-related genes (ATG) firmly regulated by numerous mechanisms involve some proteins such as ATGs, Beclin-1, LAMP-2,

BH3, Bcl2, AKT, p53, class I P13K, class III P13K and regulate release of HMGB1 from cancer cells (Su et al. 2015 and Ghavami et al. 2014).

Autophagy play a crucial pro-survival role during starvation or cell stress during growth factor deprivation, also control cell differentiation, cell survival and death that respond to extracellular or intracellular stress by degrading intracellular macromolecules and provides energy for cell functioning under the condition of nutrient depletion, however, overexpression of autophagic protein may result in cell death (Ouyang et al. 2012). In the primary step of cancer cells, autophagy get betters the fitness of cancer cells beneath stressful circumstances and work against apoptosis and necrosis (Su et al. 2015). In early stages of cancer, autophagy plays a defensive role. It has tumour suppressor role by activating pro-autophagic genes besides blocking anti-autophagic genes in oncogenesis as well (Ouyang et al. 2012), and antagonises metastasis by restricting tumour necrosis and subsequent immune cell infiltration (Su et al. 2015). In the other hand, autophagy can play a pro-tumour function by regulating a number of pathway such as Beclin-1, Bcl2, PI3K class III and I, mTORC1/2 and p53 (Ouyang et al. 2012). Overall, autophagy has two roles for cancer cells, the first role is anti-metastatic roles, during the early stages of metastasis, and autophagy inhibits the metastasis by restricting tumour necrosis and oncogene-induced senescence. The second role is pro-metastatic roles by make easy the self-alteration of pro-metastatic cells, the aloofness of ECM induces autophagy and defends disseminating cancer cells as of anoikis, facilitate cancer cells in adverse situations into dormancy and autophagy is dangerous for CSCs preservation and drug resistance (Su et al 2015).

The oxidative stress described as a situation in which oxidation exceed the antioxidant systems and to a defeat the balance between these systems. The oxidative stress plays a significant role in the intonation of messengers that adjust necessary cell membrane functions in which are very important for continued existence (Yoshikawa and Naito 2002). The oxidative stress induces senescence and autophagy in a diversity of mammalian cells, such as glioma and lung cancer (Luo et al. 2011).

The aim of this study is investigate the effect of C_{60} fullerene carbon nanoparticle on autophagy and apoptosis pathway in Human malignant glioma U-373 cell line, and crosstalk between apoptosis and autophagy under the effect of C_{60} fullerene.

1.2. Glial cell

Brain composed of cerebrum, cerebellum and brainstem. Brain made up of two special cells such as nerve and glial cells. Nerve cells take less than 10 percent of the brain. Nerve cells comprise body, axon and dendrites, which transmit the information through an electrical or chemical signal. The glial cells and supportive cells made up the other parts of brain mass, which provide protection, nourishment and support nerve cells; glial cells 10-50 times more than nerve cells and the most common types of brain tumour (Bickle 2003; Carter 2009 and Friedman and Liau 2013). Glial cells are astrocytes, which transport nutrients, hold and make the nerve cells stable in their places, regulate blood-brain barrier and digest dead neurones. Glial cells divide to microglia and macroglia cells. Microglia cells provide some function like breakdown dead neurones and pathogens. Microglia consist of oligodendroglia provide myelination to neurone cells, ependymal cells line the ventricles and secret cerebrospinal fluid (Bickle 2003 and Friedman and Liau 2013). Brain tumour arises from uncontrolled abnormal cell growth, commonly arises from macroglia cells, which called glioma or astrocytoma (Abukhdeir and Park 2009).

1.3. Glioma

Glioma is the most intracranial malignant, primary brain tumour, excessive mortality, more aggressive and more resistance to chemotherapy and radiotherapy, which arises from star-shaped glial cells, called astrocytoma (Koev et al 2010 and Jones and Holland 2001). Although most glioma is scattered, genetics has some role in gliomagenesis. Family members with a history of glioma have a 5% possibility of developing a glial tumour (Wen and Kesari 2008 and Yanhong et al, 2010). Glioma especially glioblastoma more malignant because of the glioma tumour cells have a great ability to disturb the anti-tumour immune response and lack of advance how glioma tumour cells modulate host immune response and neutralise immune-based therapy (Dix et al. 1999).

1.3.1. Glioma classification and grading

Brain cancers divide to a primary brain tumour and metastasis brain cancer. Primary brain tumours are arise from brain macroglia cells and have four grade, but metastasis brain cancer comes from the another part of the body for example lung cancer or breast cancer spread throughout the body, migrate and infiltrate to the brain throughout blood stream and form a metastasis brain cancer (ABTA 2015). In 1979, the World health organisation (WHO) classified central nervous system tumours into four grades according to histological and immunohistochemical (Jones and Holland 2011). Glioma divides according to grades, meaning related to the rate of growth and interfering brain function (Friedman and Liau 2013). The grade I glioma, it's benign slow growing and restricted glioma, as compared to high-grade glioma much better prognosis and ability to control it with a section or removing the tumour by surgery. The grade II glioma infiltrate into brain tissue and illustrate moderate strategy of proliferation, second-grade glioma nearly 70% percent have the talent to progress to high grade (grade IV) glioma over time particularly in young populace within 5-10 years after diagnosis (Jones and Holland 2011). The third grade (grade III) called high-grade or malignant astrocytoma such as anaplastic astrocytomas, anaplastic oligodendrogliomas and anaplastic oligodendroastrocytomas. The two and third grade are increased magnitude of cellularity and increased cellular atypia. The fourth-grade glioma (astrocytoma) called glioblastoma, it is malignant, extremely aggressive, the most widespread and deadly tumour in central nervous system, grade four characterised by increased cellularity as well as endothelial proliferation and necrosis (Abukhdeir and Park 2009 and Jones and Holland 2011). Amantini with colleagues (2007) classified glioblastoma U373 into grade III glioma or astrocytoma. Gliomas are divided into two major classes according to their ability to invasion and progression for malignant forms, the first classes are belong to astrocytoma grade II (WHO), anaplastic astrocytoma (WHO grade III) and WHO grade IV glioblastoma multiform. This group is generally characterised by the high ability for invasion, infiltration and significant for tumour progression. According to Wares et al (2003) described the second class is belong to these tumours that have more limited invasion potential and low ability to malignant transformation, these class include pilocytic astrocytoma (Grade I) WHO, pleomorphic xanthoastrocytoma, and subependymal giant cell astrocytoma.

1.4. Glioblastoma

Glioblastoma is a primary brain tumour, highly combative brain tumour most common in adults and among most lethal cancer in human, classified by the World health organisation (WHO) as class IV astrocytoma according to malignancy based on the histological characteristic. Glioblastoma survives apoptosis and has a resistance against available therapeutic closes such as radiation, chemotherapy and surgery; these patients will be dying not more than 14.6 months (Aleksi and Rolf 2014 and Camilla and Martha 2010). In the United States, more than 22,000 Peoples have diagnosed with malignant glioma annually and mainly will die with two years after diagnosis of diseases (Dix et al. 1999). Recently glioblastoma cancer-initiating cells have the ability to stand up against T-cells proliferation and activation inhibits induced regulatory T-cells and trigger T-cells apoptosis (Wei et al. 2010). Glioma cells could produce suppressive factor, which inhibits locally activated T-cells, thus lymphopenia observed in malignant glioma patients (Koev et al. 2010).

1.4.1. Biological behaviour of glioblastoma

Glioblastoma has two different types according to morphological differences and heterogeneity, primary and secondary glioblastoma. The primary glioblastoma is a highly aggressive with a high-grade glioma tumour which frequently affects elderly around sixty years old, in this type of glioblastoma typically mutation occurred in epidermal growth factor EGFR, cyclin-dependent kinase inhibitor 2A (CDKN2A) and loss of heterozygosity (LOH) on chromosome (10q23), which regulate phosphatase and tensin homolog PTEN gene (Krakstad and Chekenya 2010). The PTEN gene is identified as tumour suppressor gene that is mutated in many types of cancer at high frequency, the (PTEN) protein produces by (PTEN) gene which acts as a phosphatase to dephosphorylate phosphatidylinositol (3,4,5)-trisphosphate, which is important because it results in an inhibition of the AKT signalling pathway. PTEN protein phosphatase activity may be involved in the regulation of the cell cycle, preventing cells from growing and dividing too rapidly. PTEN is one of the targets for drug candidates such as the oncomiR, MIRN21 (Wang et al. 2015).

The second type of glioblastoma suffers the younger person around forty-five years old, described as low-grade glioma with slow progression approximately four to five years. The second type of glioblastoma has a mutation in platelet-derived growth factor receptor (PDGFR) and TP53 genes (Krakstad and Chekenya 2010). Every secondary glioblastoma has a mutation with isocitrate dehydrogenase-1 (IDH1) or isocitrate dehydrogenase-2 (IDH2). The 10% of primary glioblastoma have IDH mutation. Secondary glioblastoma has a higher rate of promoter methylation rather than primary glioblastoma (Goodenberger and Jenkins 2012). Recently mutation found in the (IDH1) in young patients with secondary glioblastoma (Parsons et al. 2008 and Yan et al. 2009). According to the cancer genome atlas (TCGT), Goodenberger and Jenkins genetically divided glioblastoma into four subtypes, which include classical, neural, mesenchymal and proneural. The classical subtype defined by the loss of chromosome 10 and paired events gain of chromosome 7. This subtype has PTEN and rare TP53 mutation and EGFR amplification and has disruption of retinoblastoma pathway, rarely observed NF1 gene, IDH1 gene and PDGFRA in this subtype (Goodenberger and Jenkins 2012). Described loss of INK4A/ARF, nestin over-expression and notch and SHH pathway activation (Agnihotri et al. 2012). Mesenchymal subtype has a dominant mutation in NF1 point, NF1 gene deletion or low level of NF1 expression. NF1 and PTEN co-mutation is common and high expression of MET and CHI3L1 (Goodenberger and Jenkins 2012), CD44 over-expression, TNF family and NFKB pathway activation (Agnihotri et al. 2012). Neurone marker genes expressed in neural subtype (Goodenberger and Jenkins 2012 and Agnihotri et al. 2012), including NEFL, GABRA1 and SYT1. The unique features of this subtype are EGFR amplification and rarely mutation of PDGFRA, IDH1 and TP53 in neural subtype (Goodenberger and Jenkins 2012). Overexpression of HER2 described also, the gene signature of neural subtype similar to normal brain (Agnihotri et al. 2012). A proneural subtype differs from secondary glioblastoma because of, with this subtype reported longer survival for patient and younger age of beginning. The genetic skins of proneural mutated in IDH1 (Goodenberger and Jenkins 2012 and Agnihotri et al. 2012), IDH2 (Goodenberger and Jenkins 2012), highly amplification of PDGFRA and PDGFRA gene expression (Goodenberger and Jenkins 2012 and Agnihotri et al. 2012). Loss of heterozygosity of 17p (Goodenberger and Jenkins 2012), mutation of TP53 (Goodenberger and Jenkins 2012 and Agnihotri et al. 2012), loss of chromosome 10 and gain of chromosome 7 are less common (Goodenberger and Jenkins 2012). Agnihotri

with colleagues (2012) showed the mutation of p53, loss of both INK4A/ARF and PTEN, proneural/progenitor marker expression and activation of HIF1A, PI3K and PDGFRA pathway.

1.5. Genetic alteration and signalling pathway in glioma

Glioma especially glioblastoma tumour cells subject some types of cellular dysfunction for survival against immune system and glioma therapies, the main subjects of glioma are cell cycle control dysfunction, growth factor overexpression and their receptors, genetic instability, apoptosis disorder, invasion and migration and angiogenesis (Nakada et al. 2011). The mutated genes in glioma involve the progressive loss of genes function, which is responsible for the control of apoptosis, cell cycles and cell migration (Collins 1998). The gene alteration or mutation in glioma reported as alteration of the p53 tumour suppressor gene, loss of chromosome 17p, 10, 9p and 19q heterozygosity, rearrangement and amplification of epidermal growth factor receptor gene (EGFR), MDM2 gene and amplification of platelet-derived growth factor receptor-A (PDGF-RA) and oncogenes N-myc, c-myc, N-ras and K-ras. In addition, a number of genes involved EGFR, components of the cell cycle RB, cdk4 and p16 and apoptosis regulator p53, MDM2, ARF and PTEN, there may be distinct variants of tumorigenesis based on mutated genes (Lang et al. 1994).

The abnormality of apoptosis is described in glioma, glioma abrogate apoptosis as well, a number of mutated genes involved in gliomagenesis, most notably p53 have roles in apoptosis (Nakada et al. 2011 and Chao et al. 2000). The p53 mutation causes to disturb the response of normal glial cells to apoptosis and would lead to overexpression of growth factor in low-grade glioma and allow more progression (Nakada et al. 2011). More expressed Bcl2 protein in glioma have the main role in anti-apoptotic features of glioma especially glioblastoma (Furnari et al. 2007).

Genetic instability is intracranial event, features of many tumours and encourages genomic damage. Genetic instability is an essential feature of low-grade glioma for progression to high-grade glioma over time (Leung et al. 2000 and Nakada et al. 2011).

p53 known as the guardian of the genome, any mutation in p53 leads to tumour progression through genomic instability (Nakada et al. 2011).

1.5.1. Cell cycle dysregulation

There are some cell cycle control pathway proteins, which effect the majority of the brain tumour, alteration of these genes consisting in gliomas which have the main effect in cell cycle control and tumours development such as cyclin-dependent kinase (CDK) and cyclin-dependent kinase inhibitor (CDKI). Control of the cell cycle checkpoint involves p16, CDK4, cyclin D1 and RB1. The major objective of mutation in glioma is cell cycle dysregulation, which demonstrated in glioma through Rb and p53 pathway. This pathway regulate cell cycle G1/S phase transition, this mutation leads to excessive and irregular cell division, the mutation of Rb and p53 is occurred through mitogenic signalling effectors phosphoinositol-3 kinase (PI-3K) and mitogens-activated protein kinase (MAPK) (Belda-iniesta et al. 2006).

1.5.1.1. P53 pathway

The p53 protein encoded by a p53 gene at chromosome 17q13.1. The p53 protein acts in response to diverse cellular stress to adjust target genes that persuade cell cycle arrest, cell differentiation, cell senescence, cell death, DNA repair and neovascularization. A p53 protein activates after DNA damage and induces gene transcription such as p21Waf1/Cip1 that function as controllers of cell cycle progression at G1 phase (Nakada et al. 2011). Some studies already mentioned the role of p53 as apoptosis promoter; p53 activated under a wide range of stress conditions like DNA damage, hypoxia, cellular senescence, encourage cell cycle checkpoint by abnormal oncogene expression, apoptosis and DNA repair. P53 induces apoptosis through activation of pro-apoptotic Bcl-2 family members Bax, BH3-onlyprotein PUMA and Bid (Nikoletopoulou et al. 2013).

The p53 protein has a vital role in cell cycle arrest after DNA damage by activating p21, start to DNA repair and induce apoptosis by overexpression of Bax (Gomez-manzano et al. 1997). In 30% of gliomas described p53 mutation (Gomez-Manzano et al. 1997 and Belda-iniesta et al. 2006) or loss of chromosome 17 or amplification of Mdm2 which

bind to p53 targeting, or by the loss of p14ARF protein which inactivates Mdm2, this gene regulates apoptosis. Responds to DNA damage, p53 allows the cell to control cell cycle progress or apoptosis (Gomez-manzano et al. 1997).

P53 mutation has a major role in low-grade glioma than high-grade glioblastoma multiform, p53 loss of heterozygosity found in glioma grade II and IV and amplification of Mdm2 described in 8-10% of high-grade glioma. Mdm2 and CDK4 are co-amplified the p53 and pRB pathways (Belda-iniesta et al. 2006). Mutation of p53 gene may affect both the G1/S and G1/M checkpoints (Nakada et al. 2011). The TP53 and p53 inactivated by magnification of its negative regulator MDM2 or by gene mutation or 17p loss. Any hypermethylation or deletion of these CDKN2A CDKN2B loci, which encode of both $p16^{ink4a}$ and $P14^{arf}$ have an effect on Rb and TP53 expression and lead to glioma formation (Shete et al. 2009 and Wrensch et al. 2009). The MDM2 gene at chromosome 12q14.3-q15, when it is over-expressed encodes a supposed transcription factor and improves the tumorigenic potential of the cell, forming a firm complex with p53 gene by compulsory to the N-terminal transactivation domain, inhibits p53 transcription (Kubbutat et al. 1997). Conversely, wild-type p53 enhance the transcription of MDM2 gene as auto-regulator feedback loop regulates the expression of p53. The $P14^{arf}$ gene, which part of CDKN2A locus on chromosome 9p21, encodes a protein that directly binds to MDM2 and inhibits MDM2-p53 mediated degradation and transactivational silencing, but p53 is negative regulator of $P14^{arf}$, thus any alteration in $P14^{arf}$ or MDM2 lead to inactivation of $P14^{arf}$/MDM2/p53 (Nakada et al. 2011). The p53 pathway has a vital role in the progression of secondary glioblastoma, 57% of p53 mutation is positioned in the hotspot codons 248 and 273; however, in primary glioblastoma mutations more regularly dispersed through all exons, with only 17% taking place in codons 248 and 273 (Biernat et al. 1997).

1.5.1.2. P21 roles

The p21 protein encoded by a p21 gene found on chromosome 6 at 6p21.2. This protein characterised cell cycle regulator, the function is to act as cyclin-dependent kinase inhibitor and promote cell cycle suppression through the inhibition of phosphorylation of cyclin-dependent kinases (Abbas and Dutta 2009). P21 is p53-dependent protein (Gomez-

Manzano et al. 1997). Expression of p21 cause cell cycle arrest in G_0/G_1, changing cell morphology and cell differentiation, however, p21 protein do not induced apoptosis (Koljonen et al. 2006).

1.6. Programmed cell death in glioma

Programming cell death is a genetically regulated process, dealing with apoptosis, autophagy and necrosis, triggered by the presence or absence of stimuli. It may balance between cell deaths with normal cells or may decide the fate of cancer cells (Ouyang et al. 2012). Originally thought apoptosis to be a form of programmed death alone, however, programmed cell death has extended to take in autophagy and necrosis (necroptosis). The programmed cell death confines malignant cells from surviving and distributing. However, cancer cells use various strategies by generating genetic or epigenetic changes to evade programming cell death (Su et al. 2015). The apoptosis, autophagy and necroptosis are kinds of programmed cell death, distinguished by their morphological changes (Tan et al. 2009 and Bialik et al. 2010). The programmed cell death evolve apoptosis as the first line (Kerr et al. 1972), autophagy as the second line (Liu et al. 2011; Chen et al. 2010; Huett et al. 2010 and Li et al. 2011) and necroptosis as third line (Wu et al. 2012 and McCall 2010).

1.6.1. Molecular mechanism of type I programmed cell death apoptosis

Apoptosis morphologically differs from necrosis by shrinkage and fragmentation of nucleus and cell, without rupture of the cell membrane. Apoptosis often deregulated in cancer, glioblastoma also has a resistant for apoptosis, and mutation in Bcl-2 proapoptotic protein is one of these reasons (Krakstad and Chekenya 2010). According to Hallmarks of cancer, the resistance to apoptosis is characteristic of cancer especially glioma and glioblastoma, defects in apoptosis-related not only to tumourigenesis but also related to the resistance to cancer treatment such as radiotherapy and chemotherapy (Lefranc et al. 2007). The well-established phenomenon of glioblastoma is evasion from apoptosis (Krakstad and Chekenya 2010).

The mutation of some genes, like PTEN, PI3K-III, mTOR, Nf-kb, Ras/Raf/MAPK, ERK kinase, MEK/ERK signalling cascades participate in the transmission of signals from growth factor receptors to control gene expression and inhibit apoptosis. PTEN mutation normally able to the expression of Raf/MEK/ERK, but suppress them because have the ability to elevate Akt levels to phosphorylates and activate Raf-1. In addition, an unusually activated PI3K/Akt pathway cause tumour cells to be resistant to cytotoxic insults as well as those associated to proapoptotic anticancer drugs. In malignant glioma, breast cancer, ovarian cancer and prostate cancer, deregulation of PTEN/PI3K/Akt pathway related with chemotherapy resistance, also inhibition of this pathway cause to more effectiveness to chemotherapy. In head and neck cancer, activated Raf/Ras pathway conferred to resistant to apoptosis, while the mTOR inhibition causes to PI3K activation and increase the expression of cell cycle regulatory and anti-apoptotic protein. After all, in glioblastoma cells, activation of Nf-kb pathway allows cells to resist cytotoxic insults; also, these changes have an effect on tumour necrosis factor, Fas and TRAIL receptors, which play a significant role in tumour confrontation to apoptosis during cancer progression and melanoma development (Lefranc et al. 2007).

The apoptosis regulates by two pathways extrinsic and intrinsic pathway in glioblastoma (mitochondrial pathway). The death receptor families are leading to initiating extrinsic pathway of apoptosis (Krakstad and Chekenya 2010). These signalling cascades (PTEN/PI3K/Akt /mTOR/NF-kB and Ras/Raf/MEK/ERK) has a significant roles in the directive of gene expression and avoidance of apoptosis, components of this pathway mutated or typically expressed in human cancer (Lefranc et al. 2007). The class of protein of NF-kB involved in stress responses, differentiation, cell proliferation, apoptosis, the significance of IKK/NF-kb and tumorigenesis in apoptosis fully observed (Ghobrial et al. 2005).

The apoptotic pathways in malignant glioma especially U-373 cell line has observed in the last decades. According to Zamin and his colleagues, Resveratrol-treatment glioma, inhibit the growth of glioma by senescence and apoptosis (Zamin et al. 2009). Resveratrol 100 mM activate caspase-3 and induces LDH release in glioma U-87 cells (Jiang et al. 2005), in glioma U-251 induce apoptosis and autophagy together, induce cell cycle arrest at G1 phase and increase Bax (Gu et al. 2009). Krakstad and Chekenya (2010), have

observed the Bcl-2 inhibitor ABT-737 used against glioblastoma tumours, induced apoptotic cell death in vivo and in-vitro by releasing proapoptotic Bax, however, less efficient in MCL-1 over-expressed cells. In the other hand, Gossypol 20mg/day can bind to BH3 anti-apoptotic Bcl-2 protein had a response but low and measurable. In malignant glioma, Akt inhibitor alone and with anticancer drugs together induced apoptosis in leukaemia (Fujiwara et al. 2007). The miR-149 inhibits caspase-2 expression, inhibition of miR-149 increased caspase-2 expression in A172 cells. While small interfering RNAs against p53 and p21 in U87 malignant glioma cell reduced caspase-2 expression, also knockdown of caspase-2 lead to downregulation of p53 and p21 (Shen et al. 2016). CEP55 Knockdown leads to decrease cell proliferation, glucose uptake and cell viability in U87 and T98 malignant glioma cell line, through decreasing the level of mTOR and Akt signalling pathway, which increases the levels of Bad, caspase-9 and p27 expression (Wang et al. 2016). Nutlin-3A 0.5-20 μM 96 hours induces apoptosis in wild-type p53 cell line U87 glioblastoma in a dose-dependent manner and increased expression of p21, cleaved capase-3, MDM2 and PUMA, but not in T89 mutated p53 malignant glioma. Also, induce cell cycle arrest through the mTOR pathway and induce senescence in U87 wild-type p53 glioblastoma (Villalonga-Planells et al. 2001). Schizandra B (Sch B) is isolated from Fructus Schisandrae, 100 μg/ml, 48 hours induced apoptosis in glioma SHG-44, through increased aspase-3 and Bax (Ling et al. 2015). Zhang et al, (2010) demonstrated that temozolomide induces apoptosis in malignant glioma through activation AMPK and inhibition of mTORC1 (Zhang et al. 2011). In U87 glioblastoma cells, MDM2 inhibitor result in G_1/G_2 cell cycle arrest and apoptosis through cyclin-dependent kinase inhibitor p21 and p53 reactivation (Costa et al. 2013).

1.6.1.1. The mitochondrial pathway of type I programmed cell death

The mitochondrial pathway generated by some signals such as DNA damage, oxidative stress or growth factor deprivation. The proapoptotic Bcl-2 members Bax and Bak undertake a conformational change through death signal activation, insert the outer membrane of mitochondria, and raise the membrane permeability channels to permit the release of cytochrome-c (Brenner and Mak 2009).

The intrinsic pathway comprises binding death ligands such as Fas ligand (FasL), TNF-related apoptosis-inducing ligand (TRAIL) and TNF-α, to the TNF receptor superfamily, this interaction pursued by the assembly of the death-inducing signalling complex (DISC), which composed of the Fas-associated death domain (FADD) protein and procaspase-8/10. DISC activates downstream effector caspase (caspase-3, 6 and 7) to encourage cell death or cleavages the Bcl-2 family member Bid to tBid to make active the mitochondria-mediated apoptotic pathway. Bcl-2 family members such as Bax, Bak, Bcl-2, and Bcl-xL manage the release of cytochrome-c by controlling mitochondrial membrane permeabilization (Su et al. 2015), second mitochondria-derived activator of caspase (Smac) and apoptosis inducing factor (Lefranc et al. 2007). The intrinsic pathway (mitochondrial pathway) directs to apoptosis under the director of mitochondrial pro-enzymes. If the extracellular and intracellular signals induced apoptosis, the mitochondrial outer membrane become permeable to cytochrome-c, and then released to cytoplasm and recruits Apaf-1 and caspase-9 to create apoptosome (Kerr et al. 1972), cytoplasmic cytochrome-c binds Apaf-1 and facilitates the auto-activation of caspase-9 and following activation of caspase-3 and downstream cascades (Krakstad and Chekenya 2010).

The key regulators of apoptosis Bcl-2 family include pro-apoptotic members (Bax, Bad, Bak, Bcl-XS, Bik, Bim, Bid and Hrk) and anti-apoptotic members (Bcl-XL, Bcl-2, Bcl-W, Mcl-1 and Bfl-1). In these cells, which prepared to die, the proapoptotic bcl-2 family members such as Bax disrupt mitochondria and causing the free of other protein that guide to caspase release and cell death. The anti-apoptotic Bcl-2 family members (Lefranc et al. 2007), (Bcl-XL, Bcl-2, Bcl-W, Mcl-1 and Bfl-1) (Ouyang et al. 2012 and Krakstad and Chechnya 2010), have critical roles in apoptosis dysregulation through mitochondrial pathway (Lefranc et al. 2007). The anti-apoptotic Bcl-2 protein up-regulated in glioblastoma, and made the glioblastoma more resistant to apoptosis cell death (Ouyang et al. 2012 and Krakstad and Chekenya 2010). While the reduced expression of Bcl-2 may endorse apoptotic response to anticancer drugs and amplified expression of Bcl-2, direct the cell to resistance to chemo and radiotherapy. The pro-apoptotic proteins are able to dephosphorylation and cleavage, also leading to their translocation to mitochondria and initiates apoptosis (Ouyang et al. 2012). The anti-

apoptotic Bcl-2 family members Bcl-2 and Bcl-XL bind and inhibit Bax and Bak, thus inhibit the intrinsic apoptotic pathway (Krakstad and Chekenya 2010).

The BH3-only molecules Bim, Bid and Bad can be activated and encourage oligomerization of p53 effectors Bax and Bak, they have proapoptotic activities and guide to release cytochrome-c and mitochondria-derived activator of caspase, while cytochrome-c, able to interact with Apaf-1, leads to caspase-9 activation, activates caspase-3, then activating the downstream caspase cascade, finally accomplishing apoptosis. However, the role of caspase-2 in apoptotic effect remain to be discovered but recorded that caspase-2 may act upstream mitochondrial permeabilization when DNA damage-induced apoptosis (Ouyang et al. 2012). Some another factor reported being involved in apoptotic regulation such as p53 and cellular inhibitor of apoptosis protein (cIAPs) (Su et al. 2015). The p53 protein is an important inhibitor of tumour and proapoptotic factor that receive by mitochondria in the intrinsic pathway of apoptosis, by activating some positive controller of apoptosis DR-5 and Bax (Ouyang et al. 2012).

The multifunctional protein Bcl-2L12 overexpressed in nearly all glioblastoma leads to apoptosis dysregulation through over-expression of aB-crystalline, which directly binds and inhibits caspase-3 (Krakstad and Chekenya 2010). According to literature, which observed in 20 patient glioblastoma biopsies, that apoptosis-related genes dysregulated and proapoptotic Bax negatively expressed (Ruano, et al., 2008). The Bcl-2 or Bcl-XL leads to increase tumour cells motility, beside to apoptosis resistance (Wick, et al., 1998).

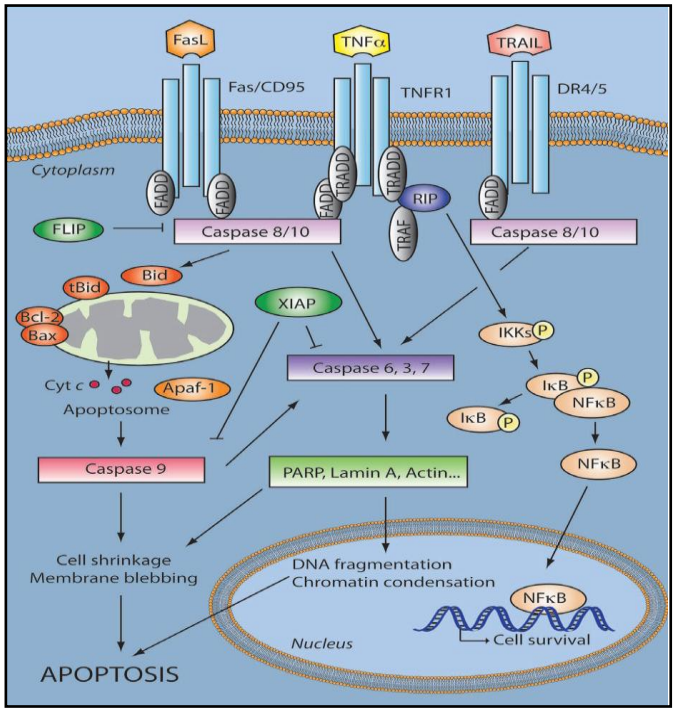


Figure 1.1. The Apoptosis signaling network in human malignant glioma. The ligand binding to death receptors TNFR1, Fas/CD95, DR4/5 be the occasion of activating the extrinsic apoptosis pathway, activate caspase cascade and finally, cleavage of cytoplasmic and nuclear substrates. TNFR1 through the NFkB may encourage survival signalling. The intrinsic pathway engages release apoptotic proteins from mitochondria, the formation of apoptosome and next caspase activation. The extrinsic and extrinsic pathways together through the caspase cascade result in cell shrinkage, DNA fragmentation and apoptosis. This pathway extremely deregulated in glioblastoma (Krakstad and Chekenya 2010)

1.6.2. Autophagy in glioma

Autophagy is a dynamic cellular process occurs as a cellular response to starvation or pathogen infection that degrades proteins or organelles. Autophagy has been involved in both immunity and development, any abnormality in this process related diseases such as cancer (Jiang et al. 2009).

Autophagy is a cellular process dependent on circumstance can encourage or block cell death. In glioma, the alkylating agent temozolomide and the mTOR inhibitor rapamycin both induce autophagy, but autophagy promotes cell death or survival remains unclear (Fan et al. 2010). The autophagic cell death may inhibit cancer metastasis, and autophagy regulates the release of HMGB1 from cancer cells to activate the dendritic cells mediated

anti-cancer immune responses. Autophagy negatively regulated by PI3K/Akt/mTOR pathway, results in the elevation of tumour stage and grade and promotes cancer progression. In the other hand, the inhibition of autophagy in a hepatocellular carcinoma (HCC) lung cancer, by silencing of beclin-1 and Atg5, decreased the pulmonary metastasis (Su et al. 2015). Some studies are co-agreed together about an autophagic role in cancer that autophagy may act as an executioner or a guardian; depend on carcinogenesis stage, surrounding cellular environment or therapy (Ouyang et al. 2012). Ultimately, Lefranc et al (2007) demonstrated, in the early stage of tumour development, autophagy act as a tumour suppressor, although autophagy suppressed in early stages of tumour progression, it seems to be up-regulated in later stages of cancer as a protective mechanism against stress condition.

The autophagic regulators inside the cells described in scientific literature, the autophagic regulators are the target of rapamycin complex-1 (mTORC1), PI3K class I, III, Beclin-1 and p53, which targeted by autophagy-related drug design (Ghavami et al. 2014). The mTORC1 described by the autophagic inhibitor, which activated by PI3K/Akt pathway. The class I of PI3K (a, b, d and g) has ability to change PIP2 into PIP3, which associate with carcinogenesis. Beclin-1 is the orthologue of Atg6; regulates class III PI3K (PI3KCIII). The ultraviolet irradiation resistant associated gene (UVRAG) and Bif-1 are positive regulator of Beclin-1, their interactions enhance PI3KCIII lipid kinase activity and facilitating autophagy, but the beclin-1 negative regulator such as Bcl-2 and Bcl-Xl, inhibit autophagy regulation by interacting with becline-1 and block becline-1 interaction with PI3KCIII (Ouyang et al. 2012).

Several proteins have been involved in autophagosome formation, beclin-1, Atg-5, 7, 10 and 12 required to the autophagic vacuole. PI3K-III is required in the early stages of autophagosome generation and Atg6 for the vacuolar formation and transport, but PI3K-I act as autophagic inhibitor-mediated through mTOR (Lefranc et al. 2007). In addition, according to Jiang et al (2009) expected some biochemical markers used to autophagic detection such as LC3-II, GFP-LC3, Atg12–Atg5, p62 and PI3K/AKT/mTOR negative regulator of autophagy.

The recent study points to autophagic pathway, the cytoplasmic constituents, including mitochondria, endoplasmic reticulum and Golgi apparatus have been proposing as the origin of the autophagosome. Although the autophagic vesicles may form through nucleation, assembly and elongation of small membrane structures result in the formation of autophagosome, after a few steps autolysosome fuse to the autophagosome and late lysosome, which has, hydrolyses enzyme degrades the cytoplasm-derived content (Chang et al. 2014). Some components are involved in both apoptosis and autophagy resistance of cancer such as (PI3K-I, Akt and mTOR) (Lefranc et al. 2007). In the autophagy pathway, two homologues Atg1 (ULK1 and ULK2), Atg13 and scaffold FIP200 protein together can form a complex, but under rich nutrient condition mTORC1 can inhibit binding Atg13 to ULK and inhibit autophagy (Ouyang et al. 2012).

In several stages, mitochondria have vital roles in autophagosome biogenesis and regulation of autophagy-mediated cell death through Beclin-1. The autophagic proteins Atg5 and LC3 employ by a mitochondrial outer membrane for elongation for the initial phagophore, lack of mitochondrial anchoring protein to the endoplasmic reticulum (Mfn2), do not show this employ. During serum starvation, mitochondria's attach together and form a tubular structure that induces autophagy, but an enlargement of senescent mitochondria during the ageing process has not the ability to fuse and autophagy inducement. Mitochondria also regulate autophagy through some proteins like Bif-1 and Sirt-1. Bif-1 (also present in Golgi apparatus) concerned in endosome formation and bind to the autophagic positive regulator. Sirt-1 encourage autophagy by direct interrelate with Atg5, 7 and LC3/Atg8. A short mitochondrial form of ARF (smARF) tumour suppressor is the other mitochondrial protein participate in autophagy induction through p53 dependent cell cycle arrest and apoptosis, however, smARF is autophagic inducer more than apoptosis. Mitochondria also participate in autophagy inducement by releasing cytochrome-c, which cleave either of Atg4 and 5 or through seep out lysosomal protease, set off activation of phospholipases and Bax/Bak (Ghavami et al. 2014). While Beclin-1 able to stimulates autophagy, inhibit oncogenesis by interacting with PI3K-III, and binding to Bcl-2, while the interaction of Beclin-1 with Bcl-2 inhibits autophagy and stimulates oncogenesis. P53 and down-regulated autophagy gene (DRAM) can activate autophagy, which mutated in 50% of cancers (Lefranc et al. 2007).

The mammalian cells have two types of p53, cytoplasmic and nucleus p53. Cytoplasmic p53 described as an autophagic inhibitor. Nucleus p53 is autophagic inducer by interacting with its objectives damage-regulated autophagy modulator (DRAM) and sestrin1/2. The DRAM is p53 target gene induces macroautophagy and trigger autophagy under p53 control. The sestrin1/2 is another target of DRAM, negative regulator of mTOR1 by activating AMPK (Ouyang et al. 2012), in the other hand autophagy promotes by AMPK protein (Ghavami et al 2014).

Glioma cells have more respond to therapy through autophagy rather than apoptosis, such as temozolomide, rapamycin and oncolytic adenovirus, which tested in patients with glioma at the Texas University; all of them induce autophagy (Jiang et al. 2009). Temozolomide (100 µM for 3 H) and Etoposide (20 µM for 8 H), temozolomide accumulates cells with DNA content at the G_2/M boundary after 3 days of treatment, Etoposide S-G_2 phase and both of them increase the level of (MAP1-LC3) and conversion of LC3-I to LC3-II. Finally, temozolomide Etoposide in glioma U-251 cells induces G_2 arrest followed by autophagy by accumulating (MAP1-LC3) protein and LC3 conversion, cell death proceeded by multi-micro-nucleation. However, temozolomide and Etoposide induced autophagic under cytoprotective effect and prevent cell death by the multi-micro-nucleation process in glioma U-251, U-373 and U-87 (Katayama et al. 2007). In the other hand, glioma treatment with Nf-Kb inhibitor resulted in autophagic cell death but not apoptosis (Koukourakis et al. 2016). Jiang et al. (2009) showed, autophagy may lead to new glioma therapies because of several lines of a proof point to glioblastoma cells appear to be less resistant to therapies that induce autophagy (Jiang, et al., 2009). Resveratrol (30 µM) for 24 and 48 hours, induce autophagy by increasing GFP-LC3 protein, acidic vesicle organelles, increases Bax expression and caspase-3 cleavage (Filippi-Chiela et al. 2011). The gefitinib (10-20 µM) treated U87 glioblastoma cells, exhibited an increase in autophagic vacuoles and cause LC3-II conversion and p62. In contrast, gefitinib-induced autophagy in U87 glioblastoma cells through alternative mechanism LKB1/AMPK by increased the phosphorylation of LKB1 and AMPK but not PARP-1 and caspase-3, and it demonstrated gefitinib-induced autophagy. However, a higher concentration of gefitinib (40 µM) caused apoptotic cell death involving PKA inactivation in glioma cells. Also in breast and lung cancer, gefitinib-induced autophagy

by LC3-II conversion, p632 degradation, GFP-LC3 conversion and appearance of cleaved-LC3 (Chang et al. 2014).

The autophagic pathway is a pathway of cell death after ionising radiation in glioma because of glioma is an apoptosis-resistant cancer cell. On the other hand, some recent study showed the glioma radio-sensitivity after suppresses the autophagy by Atg5 suppression or using 3-methyadinine. The temozolomide (TMZ) (100 μM) treated malignant glioma-induced autophagy rather than apoptosis by the accumulation of LC3 protein. In addition, TMZ increases the glioma sensitivity to autophagy after exposed ionising radiation. However, glioma cells treated with temozolomide and early stage autophagic inhibitor 3-Ma prevents the accumulation of autophagosome and suppressed the cytotoxic effect of TMZ (Koukourakis, et al., 2016). Aoki et al (2007) demonstrated in glioblastoma U87 and U373 treated with curcumin, encouraged G_2/M cell cycle arrest and autophagy not apoptosis, by inhibited the Akt/mTOR/p70S6K pathway and activate ERK pathway and causing to autophagy cell death.

Inhibitor of mTOR, PI3K-I and Akt pathways might be able to increase the sensitivity of apoptosis resistance cancer to both apoptotic and autophagic drugs, for example, cell treated temozolomide with this pathway inhibitor increase autophagic cell death (Lefranc et al. 2007). As demonstrated Lefranc and Kiss (2006), proposed using temozolomide as chemotherapy against glioblastoma, because it acts as mTOR, PI3K Akt pathway and EGFR and PGFR receptors inhibitor. Also, they demonstrated, temozolomide is a cytotoxic agent for cancers especially glioma, which produce O_6-methylguanine in DNA, which mispairs the thymine during the next DNA replication, cycle and enhances cell cycle arrest at G_2/M phase, will eventually die from autophagy.

Fan with colleagues (2010) showed the PI3K and mTOR are individually central to survival and to autophagy. In wild-type PTEN glioma and PTEN mutated glioma (U373 and U87), a dual inhibitor (PI-103) of PI3-K and mTOR, induces autophagosome formation measured by LC3 and GFP-LC3. In addition, PI-103 induces the independent LC3 conversion and degradation of p62 protein. Inhibition of mTORC-1 and 2, blocks glucose uptake, slowing tumour growth and induced autophagy as a survival pathway. Kanzawa et al (2005) showed Glioblastoma U-373 treated with As_2O3 induced

autophagy rather than apoptosis. In U-373 malignant glioma treated with As_2O_3 (4 µM) for 96 hour, increased GFp-LC3, autophagic cell death associated with mitochondrial dysfunction but not involve caspase activation and As_2O3 unregulated BNIP3 and BNIP3L genes which related to autophagic cell death and mitochondrial damage, but BNIP3 induces autophagic cell death, not BNIP3L. In the other hand, Kanazawa with colleagues (2004) observed that Temozolomide 100 µM induce autophagy in malignant glioma (U-373, T98G, U-251, GB-1, U-87 and A172), also increase GFP-LC3, acidic vesicle organelles (AVOs) autophagic characteristic, inhibit cell viability and G_2/M cycle arrest.

1.6.3. Crosstalk between apoptosis and autophagy in glioma

Apoptosis and autophagy are well-controlled processes, and they have a complex crosstalk together. Both of them apoptosis and autophagy are activated in response to energy metabolism, growth factor depletion, starvation, start LKB1/AMPK pathway and stability of p27 (Nikoletopoulou et al. 2013). P27 increases cell survival under growth factor deprivation and metabolic stress through LKB1/AMPK pathway (Maiuri et al. 2007). In another hand, downregulation of p27, activate apoptosis. A Recent study demonstrated some complex interplay between apoptosis and autophagy. Autophagy and apoptosis cooperate antagonise or help each other (Nikoletopoulou et al. 2013). However, apoptosis and autophagy both of them had a distinct morphological characteristic and physical process, but they exist complicated interrelationships together, different according to circumstances, from time to time apoptosis and autophagy able to exert synergetic effects or autophagy activated only when apoptosis suppressed (Ouyang et al. 2012). The pro-survival effect of autophagy against apoptotic cell death in gliomas has been documented (Chang et al. 2014).

The nuclear enzyme PARP1 is modulating DNA repair, transcriptional regulation, genomic stability and chromatin modification. Over-activation of PARP1 leads to energy depletion, in that way inducing necrotic cell death and stopping energy-dependent apoptosis. Unfortunately, PARP1 interferes with autophagic promoter signalling pathway such as AMPK as a cellular energy biosensor, activated during ATP depletion. AMPK promotes autophagy through activation of ULK1 or inhibition of mTOR signalling

pathway. Therefore, PARP1 elicits opposing functions by induction of necrosis due to ATP depletion and at the same time protect the cell from dying by inducing autophagy (Nikoletopoulou et al. 2013). In PTEN mutated glioma in vivo and in vitro exposed to autophagosome maturation inhibitor bafilomycin A1 (Baf-A1) and inhibitor of PI3-K and mTOR by PI-103, induces cell death by apoptosis, measured by DNA fragmentation, G1 fraction, cleavage of caspase-3 and PARP (Fan et al. 2010).

The autophagy may compensate the loss of extrinsic signals that maintain nutrients and energy metabolism for extracellular matrix (ECM)-disrupted cells, results to delay the apoptosis and providing the additional time to cells re-attach to a suitable extracellular matrix (ECM). The autophagy may support the survival of HCC through the activation of mitochondrial B-oxidation and intracellular ATP production under hypoxic condition. The cancer cells die of anoikis by the loss of ECM attachment, but the activation of prosurvival signals PI3K, Ras–ERK, NF-κB, and Rho GTPase often occurs in cancer cells and provoke anoikis, this resentment can be accomplished via the autocrine secretion of growth factors or the overexpression of receptor tyrosine kinases. Accumulating evidence proposes that autophagy also supplies a mechanism for matrix-detached pre-metastatic tumour cells to avoid anoikis (Su et al. 2015). Atg5 can enhance autophagy; knockdown of Atg5 eliminates autophagy and decreases the occurrence of apoptosis. The Atg5 may trigger to apoptosis in two ways either the 33-kDa Atg5 protein is cleft by calpains to 24-kDa under lethal stress and losses the ability to induce autophagy, this fragment going to induce mitochondrial outer membrane permeability or Atg5 may directly bind to FADD through C-terminus, thus rouse caspase-dependent cell death (Maiuri et al. 2007).

A recent study has shown that mitochondria act as an autophagic promoter through mitochondrial suicide process Mitoptosis that occurs because of mitochondrial outer membrane permeabilization, after releasing Bax and Bak a mitochondrial intermembrane space protein (DDP/TIMM8a) is released into a cytoplasm and bind to Drp1 then activates mitochondrial fission and Mitoptosis. The programmed destruction of mitochondria by mitochondrial dysfunction and ROS production direct the induction of autophagy, the recent studies point to abolition of broken mitochondria may happen each through autophagosome formation through special mitochondrial autophagy (mitophagy)

or through Mitoptotic, then released into the extracellular space by exocytosis (Nikoletopoulou et al. 2013). The mitochondrial protein involved in autophagy induction smARF (a short mitochondrial form of ARF) is tumour suppressor protein that induces cell cycle arrest through the p53 dependent pathway and types one programmed cell death apoptosis. However, smARF induces autophagy-mediated non-apoptotic cell death by knockdown of ATG5 or Beclin-1 (Ghavami et al. 2014).

The mTOR plays as a link between apoptosis and autophagy, in some types of cancer, mTOR inhibition induces apoptosis, while they activate autophagy in another setting (Iwamaru et al. 2007). By inhibiting PI3K/Akt/mTOR pathway may induce apoptosis of cancer cells by increasing autophagic flux. Autophagy antagonises apoptosis and apoptosis-related caspase reduces autophagic process, however under certain circumstances apoptosis triggered by autophagy through the activation of casase-8 and depletion of endogenous apoptosis inhibitor (Su et al. 2015).

The Beclin-1 acts as a vital regulator of apoptosis and autophagy such as, Beclin-1 have a Bcl-2 homology BH3 region, which binds with Bcl-2/Bcl-XL protein and inhibits the anti-apoptotic Bcl-2 protein or activates pro-apoptotic Bcl-2 family members like Bax and Bak (Nikoletopoulou et al. 2013 and Maiuri et al. 2007). Any mutation in BH3 domain of Beclin-1 or receptor domain of Bcl-XL interrupts the interaction between Beclin-1 and Bcl-XL, thereby lost the ability autophagy inhibition (Nikoletopoulou et al. 2013). Another BH3-only protein (BNIP3) in malignant glioma treated with ceramide enables to induce autophagy (Maiuri et al. 2007). In the other hand, deletion or depletion of BH3-only protein Bad decreases the autophagy induction through starvation, also the addition of BH3-mimetics or Bad overexpression induce autophagy in human cells (Nikoletopoulou et al. 2013 and Maiuri et al. 2007). BH3-only peptides also can acts as an apoptotic inducer through activation the mitochondrial outer membrane permeabilization and releasing Bax or Bak, or by inhibiting the anti-apoptosis protein Bcl-2 and Bcl-XL or the homologues of them. The chemotherapy ABT-737, trigger autophagy by troublemaking the communication between Beclin-1 and Bcl-2 or Bcl-XL (Maiuri et al. 2007). The interaction between Bcl-2 and Beclin-1 decrease the capacity of Beclin-1 to trigger autophagy, on the other hand, Beclin-1 contains BH3-only

proapoptotic protein cannot induce apoptosis as a result of these interactions (Nikoletopoulou et al. 2013).

The role of p53 have been observed as autophagic promoter via activation AMPK the mTOR inhibitor (Nikoletopoulou et al. 2013 and Maiuri et al. 2007), transactivate damage-regulated autophagy modulator (DRAM) which induce autophagy, tumour suppressors tuberous sclerosis-1 (TSC1) and TSC2 (Maiuri et al. 2007). In the other hand, additional activities of cytoplasmic p53 trigger apoptosis and inhibit autophagy. Deletion or depletion of cytoplasmic p53 induces cytoprotective autophagy under hypoxia condition or nutrient depletion (Nikoletopoulou et al. 2013). Recent investigation demonstrates a new interaction between apoptosis and autophagy. The interleukin-3 (IL3) growth factor induces autophagy as a prosurvival mechanism subsequently apoptotic cell death if growth factor depletion is continued. Apoptosis induction in the lead of depletion in growth factor; related to cleavage of Beclin-1 and PI3K through caspase and damages the autophagic role of Beclin-1. The caspase-dependent cleavage of Beclin-1 and PI3K trigger mitochondrial-mediated apoptosis either intrinsic or extrinsic, then lead to release proapoptotic factor. The pro-apoptotic factor Bax shrinks the autophagy by improving caspase cleavage Beclin-1, as well as Bcl-XL, enable rescue Bax-induced autophagy. Finally, apoptosis can hold back autophagy (Nikoletopoulou et al. 2013 and Maiuri et al. 2007). However, caspase-3 may rouse Atg4D-mediated autophagy to give confidence the continued existence of starved cells, because of caspase-3 cleave Atg4D, a cysteine protease that in turn cleaves the C-terminus of newly synthesised ATG8. Fortunately, overexpression of Atg4D enhances apoptosis in human cells. Indeed, apoptosis and autophagy depend on caspase activity have antagonised action and important implications, because of caspase cleavage of Beclin-1 and Atg5 modulate mitochondrial apoptosis pathway, also cleavage of Atg4D increases autophagic activity (Nikoletopoulou et al. 2013).

The crosstalk between apoptosis and autophagy demonstrated in malignant glioma by some researchers. Glioma treated with TMZ and bafilomycin late step autophagy inhibitor by preventing the fusion of mature autophagosome with a lysosome, induces apoptosis rather than autophagy (Koukourakis et al. 2016). Fan et al (2010) demonstrated that restoring of p53 by gene therapy PDGFR inhibition and anti-EGFR caused apoptotic

cell death in apoptosis resistance malignant glioma. In glioma cell treated with Resveratrol, the growth of glioma inhibited by senescence and apoptosis (Zamin et al. 2009). Resveratrol 30 µM 24 and 48 hours induced autophagy by increasing GFP-LC3 protein, acidic vesicle organelles and increases Bax expression and caspase-3 cleavage, but after treated the cells with Resveratrol and autophagy inhibitor 3-MA, Bax expression and caspase-3 cleavage increased. It's mean Resveratrol enhanced apoptosis by itself but autophagy inhibits apoptosis and apoptosis enhanced after autophagy inhibition (Filippi-Chiela et al. 2011). Pointe to Biasoli et al (2013), demonstrated that retinoblastoma protein (Rb) knockdown in glioma cells (U-87 and GBM-95)-treated VP-16 (chemotherapy drug) induced p53 non-dependent apoptosis, increased p62 protein, increased DRAM protein autophagy regulator and necessary for apoptosis, increased DNA double-strand break and reduced VP-16-induced autophagy, however, the level of LC3-II and acidic vesicle organelles increased.

1.7. C_{60} fullerene

Fullerenes are C_{60} and other discovers including C_{70}, C_{72}, C_{74}, C_{76}, C_{78} and C_{84}. They are a hollow cage with 7-10 Å in diameter, have a high molecular weight C_{60} can solubilize in lipid bilayer cell membrane (Zhou 2013). Fullerene was discovered experimentally in September 1985 at Rice University, Huston by a group included Richard Smalley, Robert Curl and Harry Kroto (Ulloa 2013). The fullerene carbon nano-particle is the first and more stable carbon isolated. Both of them, the inducer of reactive oxygen species and protector against reactive oxygen species are documented for pristine C_{60}. The toxicity of pristine C_{60} increases with organic cosolvent or surfactant. The C_{60} high affinity to chemicals, permeate it to penetrate the biological cell membrane and interfere with metabolic process inside cytosol. In addition, C_{60} can penetrate many types of tissues, neuronal cells and can accumulate in some organs such as the brain. The fullerene nanoparticles act as neurone protector and antioxidant (Baldrighi et al. 2016).

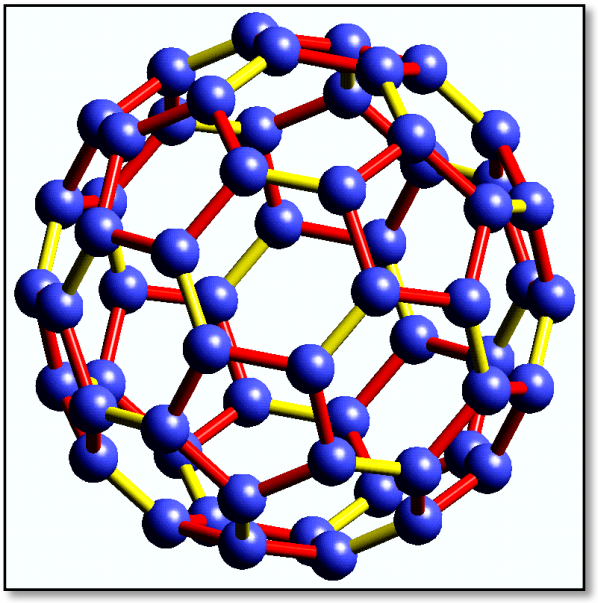


Figure 1.2. Schematic representation of C_{60} fullerene nanoparticle (Ulloa, 2013)

1.7.1. C_{60} fullerene and cancer

Nanomaterials have a large application in scientific and medical researchers because of their external dimension, show higher chemical and physical activities (De Stefano et al. 2012) doesn't aggregate in high rate, high shelf life rate, uptake by non-healthy cells compared to normal cells, induce abnormal apoptosis, proinflammatory effect and augment oxidative stress (Panzarini et al. 2013). Fullerene C_{60} has a potent anticancer (Prylutska, et al., 2014 and Panzarini, et al 2013), cytoprotective and cytotoxic effect (Panzarini et al. 2013). Fullerene C_{60} has not effects on normal cells and tissue at low concentration, have free radical scavenging, antioxidant potential and protective effect against doxorubicin-induced chronic cardiotoxicity and hepatotoxicity (Prylutska et al. 2014). Particularly, nanocrystalline C60 has cytotoxic effect against resistant chemotherapy glioma through oxidative stress-dependent and independent (Panzarini et al. 2013).

Fullerenes are more important for drug design because of their spherical shape and one nanometre in size. In addition, biologically fullerenes are influential antioxidant, can hunt ROS and reactive nitrogen species RNS (Zhou 2013). The nanomaterials can enter the cells through phagocytic and non-phagocytic mechanisms; the nanotechnology has the

significance of autophagy and lysosomal dysfunction in both of these cases (Stern et al. 2012). C_{60} fullerene has anticancer activity against rat colorectal cancer, murine hepato-cancer and mammary carcinoma (Prylutska, et al., 2014). Fullerene and its derivatives have been used as an effective scavenger for reactive oxygen species (ROS) (Wang et al. 2014). C_{60} reduces cancer proliferation, results of generating (ROS) when photo-excited, reduce tumour growth in vitro by oxidative stress, and ROS-independently slow down the cancer cell proliferation probably by autophagic degradation a number of intracellular proteins. Interestingly, the normal cells resistant to nano C_{60}-mediated ROS-independent proliferation block, Nano-C_{60} 1µg/ml increase the intracellular ROS production in B16 melanoma, nano-C60 in vitro effect the melanoma cells by blocking the cell cycle cells at low doses, but at high doses induce oxidative stress and apoptotic/necrotic cell death at higher doses. In the other hand, nano C_{60} has an opposite effect of melanoma tumour in an animal model after 14 days inoculated into animals, which treated with nano C_{60}, the tumour significantly enhanced by C_{60} instead of reduction. In addition, in spleen melanoma nano C60 induce NO production and suppress the splenocyte proliferation (Zogovic et al. 2009). Recent results point to C_{60} fullerene with doxorubicin makes a complex and may alter the anti-tumor effects of doxorubicin. However, Doxorubicin inhibits cancer cells growth with C_{60} more than Dox or C_{60} separately. C_{60} 2.5 mg/kg with doxorubicin acts as an anti-tumor agent and Dox-induced cytotoxicity (Prylutska et al. 2014).

1.7.2. The role of C60 fullerene on apoptosis and autophagy in glioma

Affect of fullerene and its derivatives whether results in apoptosis and autophagy pathway in cancer and normal cells described previously. Nano-materials cause autophagic dysfunction associated with obstruction of autophagy flux, by inhibiting lysosomal enzymes or lysosomal oxidative stress, osmotic swelling, or lysosomal membrane disruption. Interestingly, nano-materials proposed as autophagic tools monitor (De Stefano et al. 2012). C_{60} nanoparticle induced autophagy in glioma cell line through LC3 puncta formation detection (Harhaji et al. 2007). Nano-C_{60} (0.5 µg/ml) induced autophagy in HeLa cell line through the expression of GFP-LC3 protein and HeLa cells treated with Temozolomide with nano-C60, contained more autophagic vacuole both autophagosome and autolysosomes (Zhang et al. 2009).

Nano C60 depending on high or low dose can be source of necrosis or chunk cancers proliferation in human glioma cell line U-251. High doses induce ROS production and low doses inhibit proliferation depends on cell cycle arrest by inducing autophagy (Panzarini et al. 2013). C_{60} nano-particle 0.5 µg/ml induced ROS and autophagy through Atg-5 gene in HeLa cell line (Zhang et al. 2009). Isakovic with colleagues (2006) displayed in glioma U-251, C_{60} increased ROS-dependent necrosis, and DNA fragment and generate oxygen radical.

Nano-materials increase generation of reactive oxygen species (ROS), leading to oxidative stress and encourage cellular damage (De Stefano et al. 2012). Nano-material C_{60} high dose causes to ROS-mediated necrosis or C60 low dose causes ROS-independent autophagy in human and rat glioma cell lines (De Stefano et al. 2012). In HeLa and MCF-7 cells, nano C60 cause cell death mediated by autophagy (Panzarini et al. 2013). The nano-C_{60} and Doxorubicin treated cells with maximum concentration 2µg/ml killed approximately about 96% HeLa cells after 24 hours. C_{60} nano-particle 1 µg/ml with Doxorubicin 2 µg/ml induced autophagic cell death about 73% in drug resistant MCF-7 cells after 24 hours; while the same concentration at the same time without C_{60} killed approximately 5% HeLa cells (Zhang et al. 2009). Wei et al (2010) demonstrated a high dose C_{60} nano-particle 2µg/ml caused cell death about 70% in HeLa cell line and 90% in Doxorubicin resistance MCF-7. Nevertheless, low dose (1µg/mL) of nanoparticle C_{60} has the same toxicity but does not lead to significant cell death. Wang et al (2014) demonstrated, C_{60} nanoparticle didn't lead to significant decreasing cell viability in A549 cell lines, but showed ROS generation dose-dependent manner and autophagy increased (Wang, et al., 2014). C_{60} nanoparticle enhanced autophagy by increasing the ROS and level of LC3-II (Wei, et al., 2010). De Stefano with his friends (2012) showed the nano-materials trigger of intrinsic and extrinsic apoptosis pathways through oxidative stress and ROS generation (De Stefano, et al., 2012).

2. MATERIALS AND METHODS

2.1. Cell culture

Tissue culture is a broad application in the field of cell biology, in mainly cases, cells or tissues have to be grown in days or one week to obtain enough number of cells to analyse. Cell culture needs a numeral special skill to protect the structure, behaviour biology and function of the cells. The basic techniques need to preserving aseptic technique, preparing media by means of the suitable characteristics, passaging, freezing and storage, on the road to recovery frozen stocks and counting. Cell culture should be doing inappropriate techniques and sterile condition. Cell culture comprises some techniques such as below.

2.1.1. Thawing and recovering cell

Vial removed from liquid nitrogen, immediately placed at 37 °C water bath (Nüve, Turkey) and stirred up vial continuously until the medium thawed as quickly as possible approximately in (60) seconds, for preventing ice crystals formation that can cause cell lysis. The top of the vial wiped with 70% ethanol (Sigma-Aldrich, Germany). Before opening, the vial dried with air and transferred to sterile 15 ml falcon tube (ISO Lab Germany) containing 5 ml pre-warmed complete growth Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L D-glucose, L-glutamate and pyruvate (Gibco by life technology, UK), which contained 10% (FBS) fetal bovine serum (Gibco by life technology, south America) and 2% Pen/Strep (10 unit/ml, 10Mg/ml) (Gibco by life technology, USA). The cells centrifuged (Hettich, Germany) 3 minutes at 1500 round per minute (rpm) at 4°C. Discarded supernatant, and then re-suspended cell gently with 3 ml fresh growth medium and transferred to 75-cm^2 flask (Sigma-Aldrich, Germany) with appropriate amount of medium. The cells incubated in humidified incubator (Esco,

Singapore), 5% CO2 at 37 °C. Checked the cell after 24 hours to ensure that cells attached to the plate and changed medium every two days or when the colour of indicator (phenol red) changed.

2.1.2. Cell splitting

The cells splitted when the confluences of cells reached to approximately 80% or 85% in the flask. The medium removed by sterile serologic pipette (Sigma-Aldrich, Germany) inside the biosafety cabinet (Esco, Singapore). And then added 3 ml Dulbecco's phosphate buffered saline (DPBS) without Ca^{+2} and Mg^{+2} (Sigma-Aldrich from Germany), and then agitate the flask till the cover all the surface of the cells for removing the dead cells and washing the attached cells from remained medium which contain fetal bovine serum albumin should stop the action of Trypsin enzyme. After washed the cells two or three times, the DPBS removed by sterile pipette, after that added enough amount of 0.25% Trypsin-EDTA (Gibco by life technology, UK) to cover the surface of attached cells, incubated for two to five minutes in 5% CO_2 humidified incubator at 37°C. The cells should be check under an inverted microscope (Nikon, Japan) for being sure that cells detached completely. The identical growth medium Added 1:3 ratio with trypsin, inside the biosafety cabinet, and then mixed the cell by sterile serologic pipette and collected within sterile 15 ml falcon tube, then centrifuged at 1500 rpm, 4°C for 3 minutes. Inside the cabinet removed the supernatant and resuspended the cell with new pre-warmed fresh growth medium (DMEM) and poured into two new culture flasks or Petri (Sigma-Aldrich from Germany), then incubated in humidified incubator with 5% CO_2 at 37 °C for 24 hours. After 24 hours, checked the cell under inverted microscope to be sure the cells attached and changing the medium one time in two days or when the colour of the indicator (phenol red) changed until the confluence of cells reached 80-85%.

2.2. Cell proliferation assay

The cells collected from culture flask counted under an inverted microscope by hemocytometer slide (MicrobHunter, Germany). The cells 6×10^3 cultured per each well in 96 well plates (Sigma-Aldrich, Germany), divided into four groups and took at least

four replications for each group. The cells incubated in 5% CO_2 humidified incubator at 37 °C overnight, for attaching the cells to the bottom of the culture well plate. In the second day, the cells treated C_{60} fullerene different concentration 0.5, 1 and 2 µmolar. The first group was controll without any chemicals, the last three groups treated with C_{60} fullerene. In the third day, after the incubation period, added 10 µlitter the first MTT labelling reagent (concentration 0.5 mg/ml) (Roche, Germany) for each well, incubated the cells in humidified incubator with 5% CO_2 at 37 °C, 4 hours, the cells should be safe from light. After that, added (100) µlitters the second MTT reagent kit solubilization reagent (Roche, Germany) for each well, allowed the plate to stand overnight and far from any light. Checked for complete solubilization of the formazan crystals and measured the SpectraMax series absorbance by microplate readers ELISA (Molecular devices LLC, USA), the measured wavelength for formazan was between 550-600 nm according to microplate ELISA reader filters, used the reference wavelength more than 650 nm.

2.3. ROS/RNS assay

Cells cultured 15×10^3 in 48 well plates (Sigma-Aldrich, Germany) overnight, treated the cells with C_{60} 0.5, 1, 2 µM 24 hours. In the second day, discarded medium gently, then added ROS/RNS detection kit (Enzo Life science, Germany) for two Hours, in the last 25 minutes added positive control and negative control ROS/RNS reagent (Enzo Life science, Germany) for positive and negative control. Discarded ROS/RNS reagents carefully, washed two times with wash buffer (Enzo Life science, Germany). Save the cells from drying and checked under a fluorescent microscope (Nikon, Japan) 20X objective lens.

The total reactive oxygen species formation (ROS) checked by fluorescent DCFD (Sigma-Aldrich, USA) in malignant glioma U-373 cells. Cells cultured, 2×10^4 cells in 24 well plates (Sigma-Aldrich, Germany) overnight, treated the cells with C_{60} fullerene three different concentration 0.5, 1 and 2 µMolars for 24 hours, then collected cells by trypsinization, centrifuged at 5000 rpm 4 minutes, discarded the medium and Trypsin. Then washed one time with cold Dulbecco's phosphate-buffered saline (DPBS) without Ca^{+2} and Mg^{+2}. Resuspended the cells with PBS (Sigma-Aldrich, Germany) contained 20

mMolar glucose (Sigma-Aldrich, UK) and 2 μMolar DCFD (Sigma-Aldrich, USA) far from light and then incubated at 37°C for 25 minutes. The absorbance of fluorescent DCF measured by a spectrofluorometer (Perkin-Elmer LS-55, USA) set at 490 nm excitation wavelength and 520 nm emission wavelength. The result analysed by GraphPad prism 5, one-way ANOVA Tukey nonparametric analysis, after different three biological replicates.

2.4. Immunoblotting (western blotting) assay

SDS-page gel electrophoresis (Bio-Rad, Singapore) is an electrical field used for separating or purifying protein according to size and molecular weight, by using polyacrylamide and bis-acrylamide (Sigma-Life science, China) for preparing the gel. The protein molecule goes from anode to cathode or vice versa according to the electric charge present on the molecules by using an electrical source PowerPack (Bio-Rad, Singapore). This technique used for determining the amount of proteins expressed or suppressed inside the cells treated with chemicals as treatment, this technique comprises some steps and techniques as below:

2.4.1. Cell treatment

Before SDS-page, the cells treated with chemicals C_{60} fullerene for 24 hours. The 7×10^5 Cells cultured in six-well plates (Sigma-Aldrich from Germany), incubated in 5% CO_2 humidified incubator at 37°C overnight for attaching the cells to the bottom of the plate. After the period of incubation, discarded the medium with a new sterile serological pipette, washed cells two or three times by DPBS, then discarded with a sterile serologic pipette. Added 3 ml of new fresh pre-warmed medium with chemicals as a treatment in different concentration, divided into four groups, the first group was a control group, added growth medium only without chemical treatment, the last three groups were treated with C_{60} fullerene different concentration 0.5, 1 and 2 μM. The cells collected after a period 24 hours of treatment.

2.4.2. Cell lysate preparation

Lysate of the cells prepared from harvested cells within microfuge tubes 1.5 ml centrifuge microfuge tubes (Sigma-Aldrich, Germany), washed the cells two or three times by cold DPBS, centrifuged at 1500 rpm for 4 minutes and discarded the supernatant. The lysis buffer (50 mM Tris (Sigma Life Science, USA) Ph 7.4, NaCl 150 mM (Sigma-Aldrich, Germany), NP-40 1% (Sigma-Aldrich, Germany), sodium deoxycholate 0.5% (Sigma-Aldrich from Germany), sodium dodecyl sulphate 0.1% (SDS) (Merck, Germany), and protease and phosphatase inhibitor 0.01% (Cell Signalling, USA)) added to microfuge tubes, which contain the cells as pellet and resuspended. The cells mixed well by 1 ml syringe two or three times, incubated the cells with lysis buffer in ice +4 °C for one hour, mixed the cells two or three times during the incubation period. Cell debris and undegraded protein particles separated by centrifugation, (14000) rpm for 12 minutes at +4 °C. Collected the supernatant contained total protein within another microfuge tubes and discarded the pellet. The UV-1800 spectrophotometer (SHIMADZU, Japan) used for detecting the absorbance of protein samples at (595 nm) by using Bradford solution (Commasie blue, Merck, Germany), absolute ethanol (Merck, Germany), phosphoric acid 85% (H_3PO_4) (Balmumcu Kimiya, Turkey) and double distilled water).

2.4.3. Electrophoresis (Sodium dodecyl sulphate polyacrylamide gel)

The protein solution boiled with sample Laemmle buffer (60 Mm Tris-HCl (Sigma-Aldrich, Germany) ph 6.8, sodium dodecyl sulfate 2% (SDS) (Merck, Germany), glycerol 10% (Fisher from USA), b-mercaptoethanol 5% (Sigma-Life Science, Germany) and bromophenol blue 0.01% (Merck, Germany)) 5 minutes at 95°C, used thermomixer (Eppendorf, Germany) for protein linearization and giving minus charge to protein molecules by sodium dodecyl sulphate. For every 100 µl of protein sample, used 30 µl sample buffer. After that, the protein solution samples loaded to polyacrylamide gel (30% acrylamide and bis-acrylamide (Sigma-Aldrich, Germany), 1 M Tris-HCl (Sigma-Aldrich, Germany), SDS, 10% ammonium persulfate (Merck, Germany), N,N,N,N-Tetramethyl-ethylenediamine (TEMED) (Sigma-Aldrich, China) nearly the same concentration of protein, for separating protein according to size. The gel was running by

giving electricity 20 mA for 30 minutes at first, raised the electricity to 40 mA by using power supply (PowerPack, Singapore) until loaded protein reached the last destination of a polyacrylamide gel.

2.4.4. Protein transfer

The protein transferred from polyacrylamide gel to membrane to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher, USA), has more affinity for protein or nucleic acid. The gel placed in transfer buffer with small agitation for 10 minutes, the transfer sandwich constructed in transfer cassette by using two pre-wetted extra-thick filter paper approximately 3 mm and soaked Polyvinylidene fluoride PVDF membrane within 100% methanol (Sigma-Aldrich, Germany). The polyacrylamide gel should be in the cathode (red electrode) side relative with membrane in the anode (black electrode) side, between the membrane and gel should be avoided the air bubble. The cassette loaded into the wet tank with transfer buffer and used electric supply 180 mA for 2 hours; the temperature of transfer buffer should be saved in +4°C. After transferring protein from polyacrylamide gel to PVDF membrane, the membrane took and stayed wet with ultra pure water or TBS-T (0.1 Tween-20 (Merck, France), 25 mM Tris (Sigma Life Science, USA), 150mM NaCl (Sigma-Aldrich, Germany), 2mM KCl (Riedel-de Haen, Germany) and adjust pH 7.4). The membrane stained with a reversible stain like Ponceau stain (Sigma-Aldrich, Germany) to be sure there were not any air bubble during protein transformation.

2.4.5. Membrane blocking

The membrane must be blocked to prevent any non-specific binding between the antibody and PVDF membrane. The membrane incubated with 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) dissolved in TBS-T solution, one hour incubated at room temperature or overnight in +4°C with small agitation. Membrane washed two times by TBS-T, first 10 min and the second 5 min.

2.4.6. Using antibody

For detecting the effect of treatment on apoptosis and autophagy pathway in glioma cells, used some antibody against some types of apoptosis and autophagy marker proteins by the different specific dilution factor. Antibody solved within 3% BSA solved in TBS-T solution then incubated PVDF membrane overnight with primary antibodies solution [Table 1]. After primary antibody, the membrane washed with TBS-T solution five times five minutes except first time ten minutes, then incubated 1 hour with secondary antibody (anti-mouse, anti-rabbit or anti-rat according to primary antibody) solved with 3% BSA solved in TBS-T solution [Table 2]. Membrane washed five times with TBS-T solution, one time ten minutes and the rest of them five minutes.

Table 2.1. The primary antibodies used in immunoblotting. Primary antibodies concentration and the origin of issued, which used for detecting the amount of desired protein inside the cells

Name of antibody	Dilution	Obtained from
anti P53	1:500	Santacruz biotechnology
anti LC3a		
anti P21		
anti BECN1		
anti Caspase-9		
anti Caspase-3		
anti-Beta actin		
anti PARP	1:2000	Abcam

Table 2.2. The secondary antibodies used in Immunoblotting assay

Name of antibody	Dilution	Obtained from
Anti-mouse	1:5000	Santacruz biotechnology
Anti-rat		
Anti-rabbit		Abcam

2.4.7. Detection

The PVDF membrane incubated with ECL solution (Abcam, UK) three minutes, and then stayed with medical film (Konica, USA) in dark place side by side by using cassette, exposure times are 3 seconds to 5 minutes according to different types of antibodies and then washed film with medical X-Ray machine (Carestream, USA).

2.5. Statistical analysis

All data from different three replicate were statistically analysed. The significance assessed by one-way analysis of variance with Tukey nonparametric test, a value of $P<0.05$ was considered statistically significant.

3. RESULTS

3.1. C_{60} fullerene decreases cell proliferation in malignant glioma U-373 cell line.

Cell proliferation assay depends on the reduction of yellow tetrazolium salts by metabolic activities of active cells, partially by the action of dehydrogenase enzymes. The resultant intracellular formazan is able to solubilized and measured by spectrophotometric means (American Type Culture Collection 2011). To examine the effect of C_{60} fullerene on cell proliferation, cell treated with different concentration of C_{60} fullerene 0.5, 1 and 2 μM, 24 hours. Cell proliferation levels are measured by MTT assay. The obtained results showed that nanoparticle C_{60} fullerene has an anti-proliferation cancer role in malignant glioma U-373 cells in a dose-dependent manner [Figure 3.1]. The fullerene C_{60} 0.5 μM significantly inhibits about 15% ± 0.1 glioma cell proliferation rate (**P<0.05), the C_{60} fullerene 1 μM considerably inhibits about 18% ± 0.1 glioma cell proliferation (**P<0.05) and C_{60} fullerene 2 μM significantly decreased cell proliferation rate approximately about 29% ± 0.1 after 24 hours treatment (***P<0.05), as shown in Figure 3.1.

To sophisticated the effect of C60 fullerene nanoparticle in different concentration 0.5, 1 and 2 μM, 24 hours on the malignant glioma U-373 cell line. The cell viability result assessed from MTT assay. The C60 fullerene has effect as anti-cell viability in a malignant glioma U-373 cell line in a dose-dependent manner after treated with C60 fullerene different concentration 0.5, 1 and 2 μM, 24 hours, as shown in Figure 3.2. C60 fullerene high dose 2 μM concentration 24 hours, more significantly inhibited the viability of glioma cell line about 29% ± 0.15 when compared to control group (***P<0.05). Also, C60 fullerene 0.5 μM decreased cell viability 15% ± 0.15 and 1 μM significantly decreased the viability of cell 21.3% ± 0.15 (**P<0.05) [Figure 3.2].

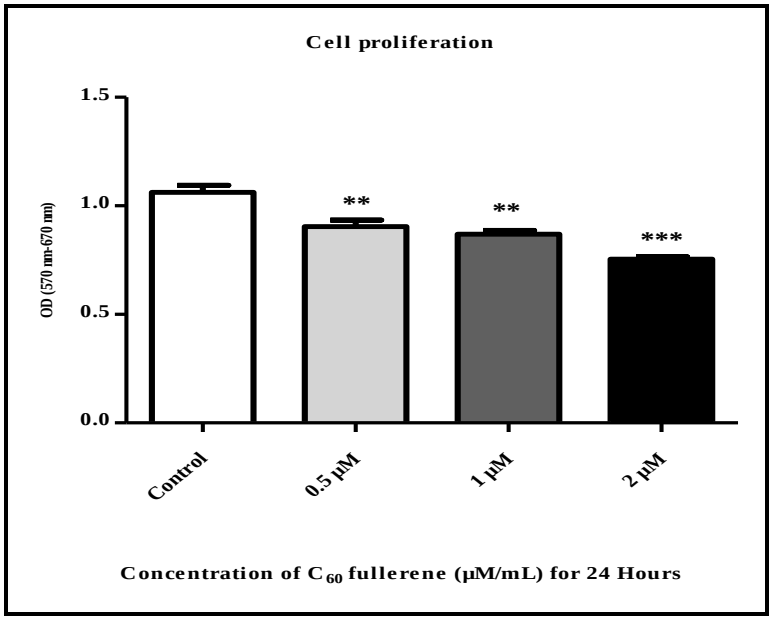


Figure 3.1. The level of glioma U-373 cell proliferation of control and treated with C60 fullerene in vitro. Seeded 7×10^3 cells and then treated with C_{60} fullerene by different concentration 0.5, 1 and 2 µM, 24 hours. The absorbance analysed by ELISA reader using two different absorbencies (A_{570} - A_{670}). The result analysed by one-way *ANOVA* GraphPad prism 5

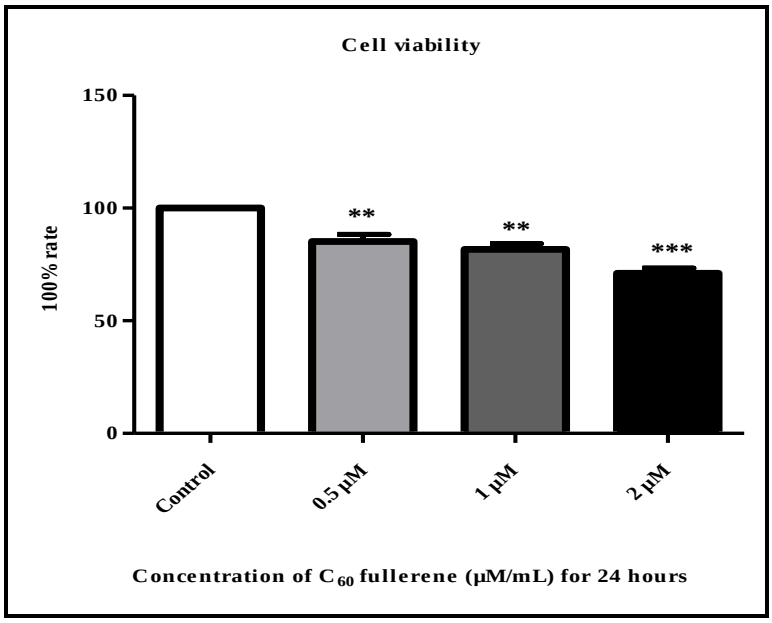


Figure 3.2. Cell viability levels of Glioma U-373 cells-treated with C_{60} fullerene. Cells 7×10^3 Seeded and then treated with C60 fullerene by different concentration 0.5, 1 and 2 µM, 24 hours. C60 fullerene decreases malignant glioma U-373 cell viability in dose-dependent manner. The result analysed by dividing the group results to the control and multiplying by 100, and then analysed by one-way ANOVA GraphPad prism 5, ($P < 0.05$, P-value 0.0001)

3.2. ROS/RNS-increased in malignant glioma U-373 cells by C_{60} fullerene

ROS/RNS assay used for oxidative stress or total ROS detection and reactive NO detection (Schneider 2015). To elaborate oxidative stress under C_{60} fullerene effects on the malignant glioma U-373 cell line, cells seeded 2×10^4 in 24 well plates and then treated with nanoparticle C_{60} fullerene in different concentration 0.5, 1 and 2 μM for 24 hours. The effect of C_{60} fullerene Analysed with fluorescent microscope 20X magnification objective lens by using green (490/525 nm) and red filters (650/670 nm).

To maintain ROS result under the effect of C_{60} fullerene was also analysed ROS by using 2,7-dichlorofluorescein diacetate (DCFD), ROS formation assayed by resuspended and incubated cells with buffer contained 2 μM (DCFD) 25 minutes in 37℃. The intracellular esterase deacetylated DCFD to form non-fluorescent dichlorofluorescin (DCF), and then DCF oxidize by intracellular ROS to form the highly fluorescent (DCF). The nano-particle C_{60} fullerene plays a role as an oxidative stress inducer in malignant cancer glioma U-373 cell line after 24 hours treatment with C_{60} fullerene 0.5, 1 and 2 μM, all of C_{60} fullerene concentration increased ROS and NO as shown in Figure 3.3. The C_{60} fullerene induced total ROS when confirmed by DCFD; all concentration 0.5, 1 and 2 μM appreciably increased total ROS in malignant glioma cell similarly 45% ±2 (***$P<0.05$), as shown in Figure 3.4.

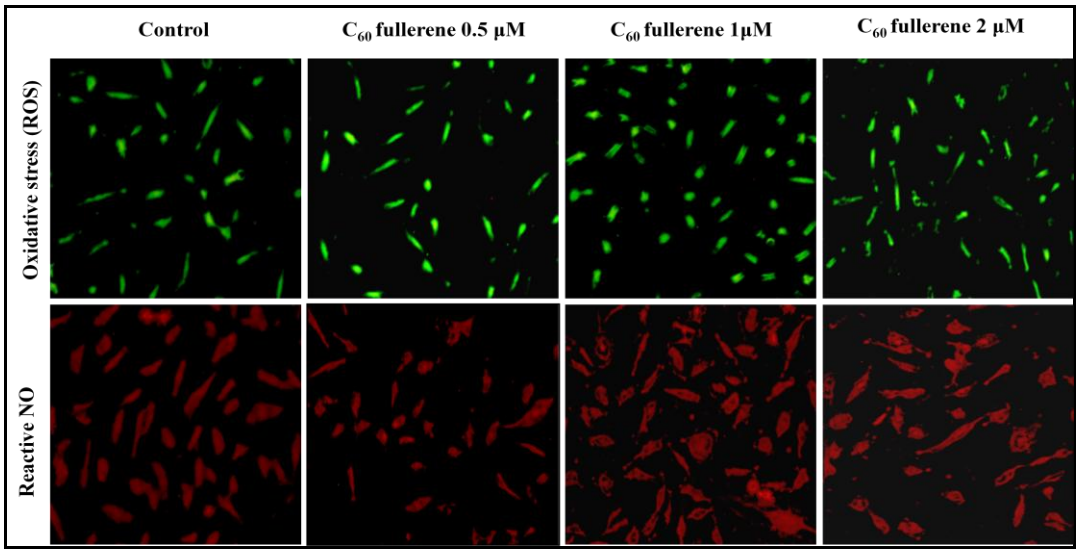


Figure 3.3. The levels of ROS and NO in glioblastoma U-373 cell line treated with C_{60}. Cell seeded in 48 well plates, treated with C_{60} fullerene different concentration 0.5, 1 and 2 µM, 24 hours. The cells treated with ROS/RNS solution (ENZO, Germany) 2 hours then checked under fluorescent microscope 20X objective lens by using green (490/525 nm) and red filters (650/670 nm). The C_{60} fullerene induced ROS and increased reactive nitric oxide (NO) in a malignant glioma U-373 cell line

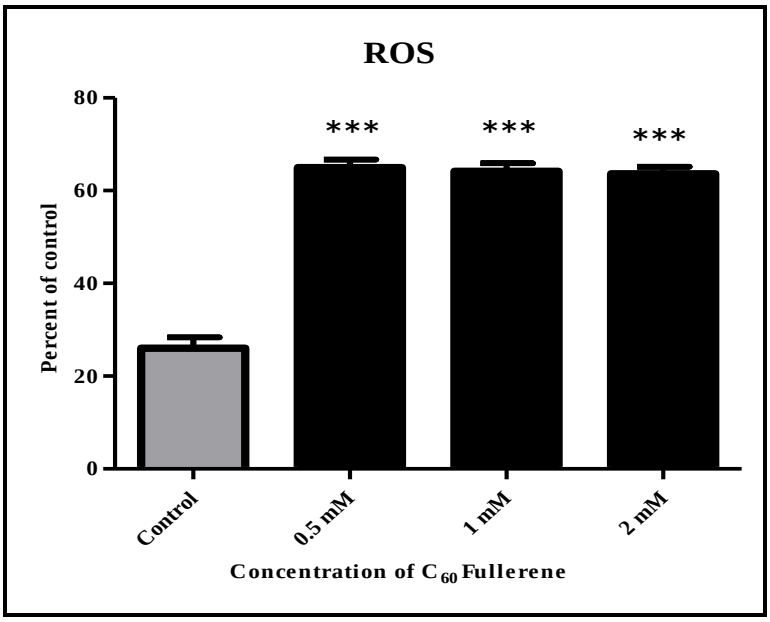


Figure 3.4. C60 fullerene induced total ROS in the malignant glioma U-373 cell line. The cells treated with C_{60} fullerene 0.5, 1 and 2 µM, 24 hours, cells harvested and then incubated with buffer contained 1.5 µM DCFD 25 minutes at 37 C. The total ROS checked by spectrofluorometer 490nm excitation and 520nm emission wavelength. C_{60} fullerene all concentration induced total ROS in malignant glioma cells about 45% ±2. Result analysed by one-way ANOVA Tukey non-parametric

3.3. The role of C_{60} fullerene in modulation of apoptotic and autophagic marker in malignant glioma U-373 cell line

The effect of C_{60} fullerene 0.5, 1 and 2 μM 24 hours complicated by using western blot for analysing the proteins amount under the C_{60} fullerene effect, after treated malignant glioma U-373 cells with C_{60} nanoparticles 0.5, 1, 2 μM for 24 hours with three different replicates.

3.3.1. C_{60} fullerene modulated autophagic marker beclin-1 and LC3 protein expression in malignant glioma U-373 cell line

To elaborate whether autophagic marker also results in the activation of autophagy pathways, we measured protein levels of the autophagic markers beclin1 and LC3, as shown in Figure 3.5.

The mammalian autophagy orthologue of yeast Atg6 gene (Beclin-1) is an necessary mediator of autophagy and has a role as a tumour suppressor. Beclin-1 mRNA and protein low expressed in glioblastoma, may point to the reduction of autophagy (Huang et al. 2010). Depending on interaction with the photoinositide-3 kinase (PI3-K), Beclin-1 has a vital role of autophagic proteins to pre-autophagosomal structure. Fascinatingly, Beclin-1 can get involved at every major autophagic pathway steps; can be autophagic inducer or inhibitor by its interaction with beclin-1 binding protein, in consequence of unstable, transient or take place only under precise condition (Kang et al. 2011). The western blot result illustrated C_{60} fullerene nanoparticle-induced beclin-1 protein expression in dose dependent manner [Figure 3.5]. C_{60} fullerene 0.5 μM induced the expression of becli-1 protein but it does not have a significant effect. The another two more higher concentration of C_{60} fullerene 1 and 2 μM induced beclin-1 protein level inside glioblastoma U-373 cells, especially C_{60} fullerene 2 μM has a vital effect on becline-1 protein expression (***$P < 0.05$) three time more than 1 μM C_{60} fullerene (*$P < 0.05$) as shown in Figure 3.5. The homologue of yeast Atg8/Apg8/Aut7, essential for autophagy microtubule-associated protein-1 light chain-3 (LC3) which has two types LC3-I cytosolic and LC3-II membrane-bound protein (Kabeya et al. 2000). In mammalian cells, LC3-II protein is more important for starting autophagy. While, the

LC3-II protein degrades rapidly by the lysosomal enzyme, during autophagy followed by autolysosome formation. LC3-II protein uses protease inhibitor and pepstatin-A during starvation-induced autophagy, which cause of lysosomal turnover after autophagosome formation (Tanida et al. 2005). The autophagy depends on Atg5/Atg7 associated microtubule-associated protein light chain 3 (LC3). Furthermore, a recent study demonstrated that Atg5/Atg7 is an independent pathway of autophagy and not associated with LC3 processing but appeared to involve autophagosome formation (Kang et al. 2011). Standard deviation means, one-way ANOVA, (Tukey nonparametric) analysis gives us a result of the conversion of LC3-I to LC3-II protein in a malignant glioma U-373 cell line when treated by C_{60} fullerene *in vitro*. C_{60} fullerene low dose 0.5 μM does not have any effect on alteration of LC3-I to LC3-II. In the other hand, the conversion of LC3-I to LC3-II considerably induced under C_{60} fullerene 1 μM treatment (***$P < 0.05$). The high dose of C_{60} fullerene 2 μMolar induced the conversion of LC3-I to LC3-II when compared to control. Nevertheless, the conversion of LC3-I to LC3-II suppressed by C_{60} fullerene 2 μMolar when compared to C_{60} fullerene 1 μMolar, as shown in Figure 3.5.

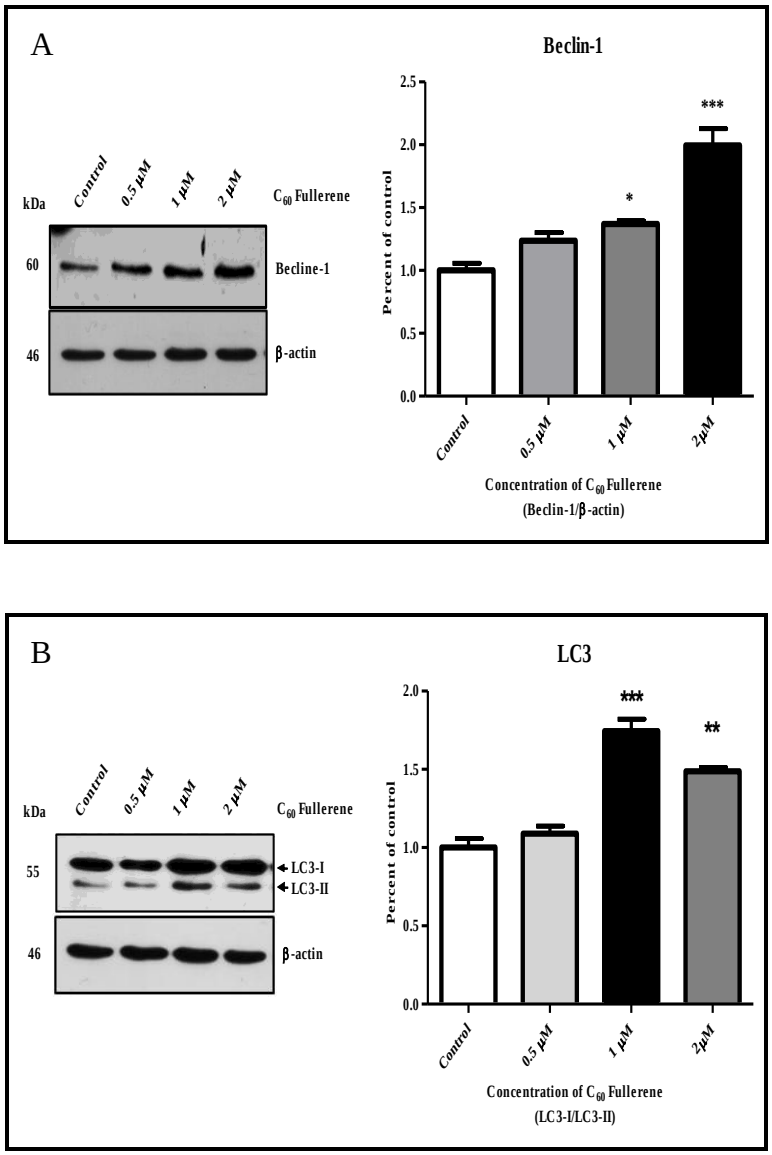


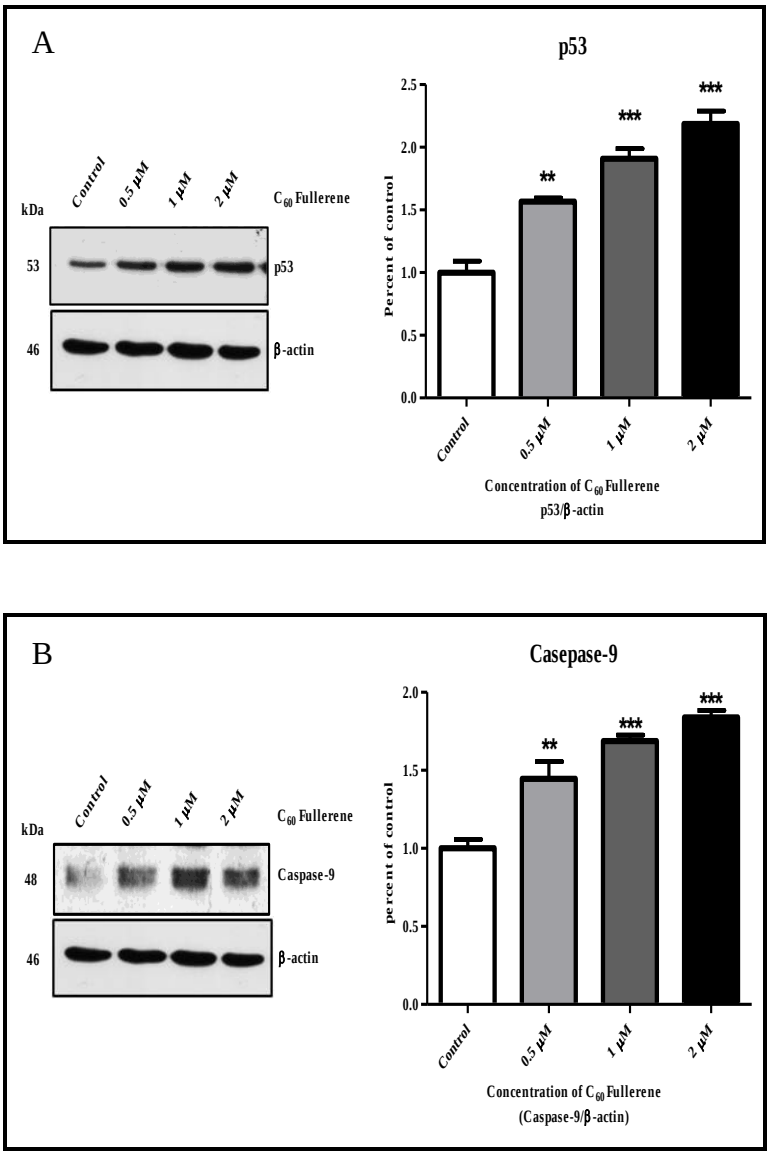
Figure 3.5. The role of C_{60} fullerene on autophagic marker protein in U-373 cell. Cells treated with C_{60} fullerene 0.5, 1 and 2 μM for 24 hours, cells harvested, and then analysed protein amount by western blotting with the indicated antibodies at the various time. A. beclin-1 protein result is analysed by extracting beta-actin and comparing to control. B. LC3-I/II protein is analysed by the conversion ratio of LC3-I to LC3-II and comparing all concentration to control. The result analysed by detecting the amount of protein (ImageJ), and then analysed by one-way ANOVA Tukey nonparametric

3.3.2. C_{60} fullerene induces the expression of apoptotic protein markers p53, caspase-9 and caspase-3 in malignant glioma U-373 cell line

To visualize the stimulation of first programming cell death (apoptosis) in human malignant glioma U-373 cell line and elaborate further expression of p53, caspase-9 and caspase-3, cell treated with nanoparticle C_{60} fullerene 0.5, 1 and 2 μM for 24 hours and then analyzed the level of apoptotic marker proteins using western blotting. The result analysed by GraphPad prism 5, one-way *ANOVA* Tukey non-parametric analysis.

P53 is the most characterised tumour suppressor protein, has a pleiotropic effect on metabolism, proliferation, antioxidant defence, senescence, genomic stability, cell death (Tasdemir et al. 2008), cellular responses to DNA damage, cell cycle regulation and apoptosis (Eisele and Weller 2013). P53 is a genetic alteration often seen in astrocytoma especially glioblastoma (Manuscript 2009). P53 as a product of Tp53 gene has an essential role in P53 induce cell cycle regulator p21 or Bax mediator of mitochondrial apoptosis (Eisele and Weller 2013). Reactivation of p53 induces autophagy in many cancer cell lines response to different treatment. Vice versa, reduction of p53 in some cancer cell lines induced autophagy (Tasdemir et al. 2008). In addition, nucleus p53 induces autophagy by mammalian target of rapamycin (mTOR) and AMP-activate protein kinase or AMPK downregulation (Maiuri et al. 2010). Western blotting analysis result showed C_{60} fullerene induced p53 protein expression in a malignant glioma U-373 cell line in dose dependent manner. C_{60} fullerene 1 μM significantly induced p53 protein expression (** $P < 0.05$). The high dose of C_{60} fullerene 1 μM noticeably induced p53 protein expression inside glioma U-373 cell line after 24 hours treatment (***$P < 0.05$). Nevertheless, the high dose of C_{60} fullerene 2 μM dramatically induced the expression of p53 protein (***$P < 0.05$). As shown in Figure 3.6. The caspase-9 protein activated during the intrinsic pathway of mitochondria (Zarnescu et al. 2008), cleaves and activates pro-caspase-3 (Karlsson et al. 2004). The caspase-9 protein expression induced in malignant glioma U-373 cell line under the effect of C_{60} fullerene in different concentration. C_{60} fullerene low dose 0.5 μM induced, when compared to control group (**$P < 0.05$) and C_{60} fullerene high doses 1 and 2 μM appreciably induced caspase-9 protein expression more than 0.5 μM C_{60} fullerene (***$P < 0.05$), as shown in Figure 3.6. The caspase-3 is activated cell protein inside the apoptotic cell through an extrinsic and

intrinsic pathway that initiates degradation of the cell in the final stages of apoptosis (Zarnescu et al. 2008). According to western blot analysis result in various treatment, caspase-3 protein noticeably induced, look like the same in both of C_{60} fullerene high doses 1 and 2 μM during 24 hours treatment (***P < 0.05). However, C_{60} fullerene low dose 0.5 μM shows not a big difference, when compared to control group (*P < 0.05), as shown in Figure 3.6.



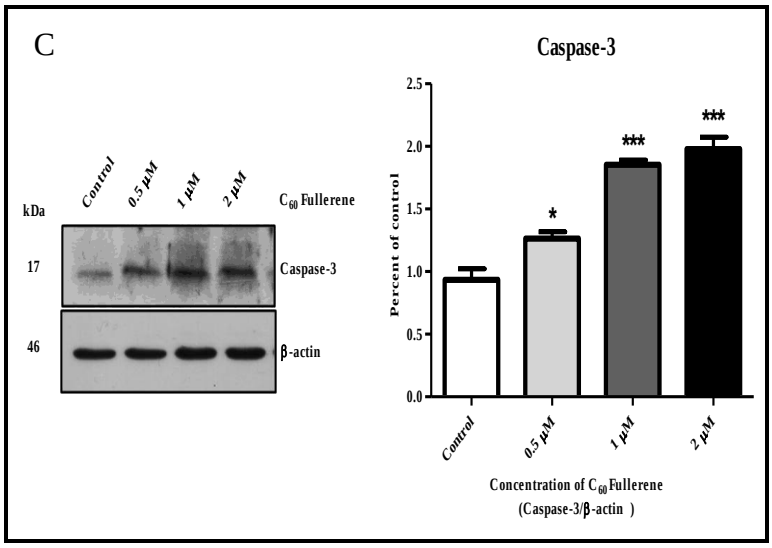


Figure 3.6. C_{60} fullerene induces apoptosis in the human malignant U-373 cell line. The human malignant glioma cells treated with C_{60} fullerene (0.5, 1 and 2) µMolars for 24 hours, harvested cells used in western blotting for detecting apoptosis protein markers, A. The p53 protein expression levels after extracting beta-actin and compared to control group (***P < 0.05), B. The caspase-9 protein expression levels extracted beta-actin protein amount (***P < 0.05), C. the caspase-3 protein expression inside the cells after extracting beta-actin protein level (***P < 0.05), showed results analyzed by one-way ANOVA Tukey non-parametric analysis

3.3.3. C_{60} fullerene modulates cell cycle arrest, PARP expression

A nuclear enzyme ~113 kDa encoded by the ADPRT-1 gene located on the human chromosome at the q41-q42 position PARP-1, can bind and damage DNA nicks (Curtin 2005 and Mounira et al. 2014). PARP-1 can bind and repair the broken single strand DNA (Tentori et al. 2014 and Mounira et al. 2014). The Poly ADP-ribose polymerase (PARP) is a nuclear enzyme divided into some structures DNA binding domain 42 kDa, catalytic domain 55 kDa (Aoufouchi et al. 1999), single strand break repair 116 kDa (Mounira et al. 2014) and less well characterised 17 kDa central domain. The nicked DNA during the early stages of apoptosis triggers the PARP, leading to ATP and ADP depletion. On the other hand, the executioner caspases role in activation cascade and cleave other proteins such as PARP. Consequently, inactivation of PARP may facilitate the DNA fragmentation (Aoufouchi et al. 1999). The expression of PARP involved in western blot analysis in different three replicate shows the effect of C_{60} fullerene on malignant glioma U-373 cell line after 24 hours. The C_{60} fullerene significantly affected

the level of PARP kind of, C_{60} fullerene low dose 0.5 μM did not has any affect of the expression of PARP ($P < 0.05$). Interestingly, the high dose of C_{60} fullerene 2 μM conspicuously increased the expression level of PARP inside glioma cell line after 24 hours of treatment when compared the control group (***$P < 0.05$). In another hand, C_{60} fullerene 1 μM induced the expression of PARP when compared to control and 0.5 μM C_{60} fullerene group (**$P < 0.05$) after 24 hours, as shown in Figure 3.7. Cell cycle regulator tumour suppressor p21 protein regulated by p53 protein. Malignant glioma U-373 cell treated with C_{60} fullerene demonstrated the expression of p21 protein induced after treated malignant glioma U-373 with C_{60} fullerene. A more significant effect come into view with C_{60} fullerene 1 μM concentration treatment which induced the expression of p21 protein inside the malignant cell line (***$P < 0.05$) and noteworthy expression of p21 protein with C_{60} fullerene 2 μM concentration (**$P < 0.05$), but 0.5 μM doesn't have a considerable effect when compared to control, as shown in Figure 3.7.

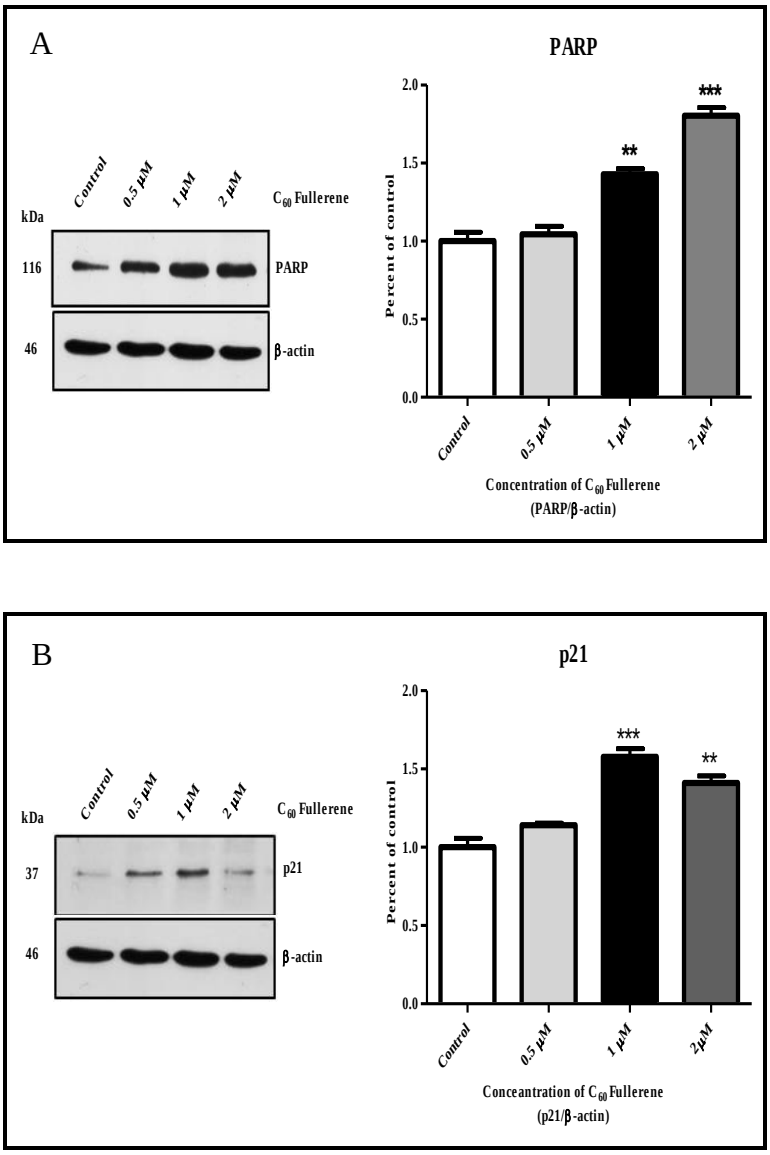


Figure 3.7. C_{60} fullerene induces cell cycle arrest and PARP expression in U-373 cell line. The human malignant glioma cells treated with C_{60} fullerene for 24 hours, harvested cells used in western blotting for detecting protein expression. A. the PARP protein levels in malignant glioma after 24 hours treatment with C_{60} fullerene (0.5, 1 and 2) μMolars (***$P < 0.05$). B. the p21 protein levels in malignant glioma after 24 hours treatment with C_{60} fullerene (***$P < 0.05$). The result analysed by one-way ANOVA Tukey non-parametric analysis

4. DISCUSSION

Glioblastoma multiforme (GMB) and alternatives are classified as WHO grade IV. It's the most malignant astrocytoma and most common brain tumour. Glioblastoma prognosis and survival of patients remain poor, and conservative forms of GBM treatment are not expected in malignant phenotype of biology because, the phenotype of brain tumour cells transformation is highly complex and the consequence of the dysfunction of a variety of internal pathways (Ray 2010). Nevertheless, apoptotic key regulators are present in glioblastoma cells, but glioblastoma is resistant to apoptosis as a result of altering some apoptotic regulator pathway or receptors (Eisele and Weller 2013). The third form carbon fullerene C_{60} Buckyball is a combination of 12 pentagonal and 20 hexagonal rings forming a spheroid shape. Due to very practical properties, has become an important molecule in science and technology. C_{60} fullerene has an antioxidant role (Ulloa 2013). C_{60} fullerene used as a carrier of specific drug delivery in cancer treatment based on absorption of UV light used as reactive oxygen inducer for enhancing ROS and allows C_{60} to be photosensitizer (Shi et al. 2016). In the other hand, C_{60} induces ROS in cancer cells, not healthy adult normal cells (Prylutska et al. 2014). Beside it, nano C_{60} enhance autophagy in cancer cells by increasing ROS, oxidative stress and LC3-II formation, also increased chemotherapeutic susceptibility (Wei et al. 2010).

In spite of the fact that, the role of C_{60} fullerene in cancer has been described the potential regulation of autophagy has not been addressed in glioma. The details about autophagy molecular mechanism remain unknown. Therefore, the effect of C_{60} fullerene on p53/caspase-9/caspase-3, p21/PARP and autophagic marker protein beclin-1/p21 investigated in human astrocytoma cell. The programmed cell death took place in a biological process mediated by an intracellular program. The programmed cell death type-I is apoptosis, distinguished by morphologically distinct characters and energy-dependent biochemical mechanism.

Apoptotic characters are cell shrinkage, smaller in size, cytoplasm dense and chromatin condensation and fragmentation. The apoptosis has two main pathway, extrinsic pathway (death receptor pathway) and mitochondrial or intrinsic pathway (Elmore 2007). A physiological occurrence upholds homeostatic function like protein degradation and organelle turnover described as second programmed cell death autophagy is quickly up-regulated under cellular stress, providing an option source for energy generation to allow incessant cell survival. Up until now, accumulating data give proof that the autophagic machinery can be employed also to kill cells under autophagic cell death named caspase-independent programmed cell death (Gozuacik and Kimchi 2007).

The anticancer effect of pristine C_{60} has been observed in many different investigational systems (Isakovic et al 2006). In this study, investigated U-373 MG cells proliferation and viability under the effect of C_{60} fullerene. Recent studies demonstrated, U-373 MG cell line, androglobin ADGB has an oncogenic function, which transfers oxygen to the brain. Androglobin knockdown inhibited all growth in the malignant glioma U-373 cell line (Huang et al. 2014). The miRNA-323-5p has an important role in cell growth and death; over-expressed in a malignant glioma U-373 cell line leads to growth and activity reduction in this cell line (Yang et al. 2015). Also, the malignant U-373 cell growth was significantly reduced, when treated with the flupirtine, which antagonises the glutamate and NMDA receptor (Panchanathan et al. 2013). The neuroprotective effect of C60 fullerene and derived of fullerene has been focused in many studies, which serves as "radical sponge" by capable of putting in multi radicals per molecule and can diminish cytotoxic effects caused by oxidative stress inside the cells (Lee et al. 2011).

The observed results in this study [Figure 3.1 and Figure 3.2], demonstrated the anti-proliferation and viability reduction effect of C_{60} fullerene on U-373 MG cell line in dose-dependent manner after 24 hours exposure. C_{60} fullerene high dose 2 μM considerably, decreased cell proliferation and viability about 29%±0.1. As demonstrated Ershova et al (2016), high dose of C_{60} fullerene significantly decreased the number of human embryonic lung fibroblasts cells. In addition, the result in this study illustrated C_{60} fullerene low dose 0.5 μM inhibited cell proliferation about 15% ±0.1, cell viability about 15% ±0.15 and C_{60} fullerene high dose 1 μM significantly reduced the proliferation and viability of malignant cell (18% ±0.1 and 21% ±0.15). The data's in this study are in a

good agreement with the recent investigation. For example, Prylutska et al (2011), demonstrated C_{60} fullerene has an inhibitory effect on the tumour growth in mice transplanted-lung cancer was observed on 11th and 13th days of the experiment, but the maximum inhibition value was found on the 23rd day of the experiment, which inhibited 76.5% of tumour cells. Also, C_{60} fullerene inhibited cancer proliferation 22.5% in Lewis lung cancer mice (Prylutska et al. 2014). In the other hand, some investigation slightly in confirm or against this study results, as demonstrated Franskevych et al (2016), slightly decrease the viability of leukemic cells treated with C_{60}. The C_{60} fullerene didn't lead to significant cell death in A549 lung cancer cell line (Wang et al. 2014). Also, C_{60} nanoparticle inconsiderably increased the proliferation of IL-2-dependent T-lymphoblastoid WE17/10 cell line after 72 hours exposure (Côté-Maurais and Bernier 2014). However, C_{60} fullerene with doxorubicin drug, increased cytotoxicity and decreased cell viability in HCT-116 colon cancer cell and MCF-7 breast cancer cell line (Panchuk et al. 2015). The proliferation inhibition of U-373 MG cells gets to maximum after treated with Limoniastrum guyonianum (400) μg/ml after 48 hours (Mounira et al. 2014).

Sun and colleague suggested that C_{60} fullerene could be a candidate for cancer therapy because of photodynamic effect to create the free oxygen species under light illumination (Sun et al. 2016). Due to their unique chemical and physical properties electronic structure, unsaturated bond and three-dimensional shape enable to cross the membrane and blood barrier, might be exploited in biochemistry and medicine areas (Wang et al. 2014). Nano-C60 described as ROS producer by photo-activation, in non-cytotoxic concentration, nano-C60 increased killing of cancer by sensitising cells to chemotherapy (Zhang et al. 2009). ROS induce caspase-independent cell death autophagy in a variety of cell types (Lee et al. 2011). The oxidative stress production ability of C_{60} fullerene have been shown in the scientific literature, as demonstrated in Ershova et al (2016), C_{60} fullerene by accumulating in cell cytoplasm increased the ROS level after three-hour exposure and up to 24 hours in human embryonic lung fibroblasts (HELFs) (Ershova et al. 2016). The obtained results in this study have a good conformity with previous and investigation in Wang et al (2014) which demonstrated C_{60} fullerene increased ROS in A549 cell line in a dose-dependent manner. In this thesis, C_{60} fullerene severely increased the ROS when analysed the DCF fluorescence intensity, all concentration of C_{60} fullerene

0.5, 1 and 2 μM after 24 hours in U-373 MG cell line in vitro, increased the level of ROS approximately 45% ±2, as shown in Figure 3.4. In another hand, as mentioned in Franskevych et al (2016), Photo-exited C_{60} fullerene induced NO production and ROS in the leukemic cell, supported this thesis result under the fluorescence microscope, C_{60} fullerene considerably increased the accumulation of total ROS and NO in the cell cytoplasm. The results of this study showed C_{60} fullerene might lead to decreased cell proliferation and viability, under the increasing of oxidative stress, ROS generation and NO production. According to the establishment in the scientific literature, Aldinucci et al (2013), NO is an important factor causing to cell damage and a significant decrease of cell proliferation. In Glioma U-373, hydrogen peroxide leads to decrease cell viability and oxidative stress generation (Nakayama et al. 2016). C60 fullerene derivative (PEG-C60-3) induced cytotoxicity in neuron-2A cells (Lee et al. 2011).

Autophagy is one of the cellular pathways, which have established more attention in recent times (Zhang et al. 2009). The survival in a stressed condition, degradation of long-lived proteins and organelles are well-known features of cellular process autophagy (Lee et al. 2011). Autophagy-related proteins Beclin-1, LC3-I and LC3-II, regulate autophagic activity. Beclin-1 protein (Atg6) is required and upstream regulator of autophagy in the initial steps of autophagy (Wang et al. 2014). In autophagy pathway, the Beclin-1 has a key function in enrollment of autophagosome membranes (Lee et al. 2011). Although, LC3 which called Atg8 is a narrative indicator for autophagy and necessary for autophagosome formation, the two form of LC3; LC3-I and LC3-II which related to autophagosome, participate in the autophagic process. While, LC3-I transform to LC3-II, the ratio between LC3-II/LC3-I related to the activity of autophagosome and use as autophagy marker (Wang et al. 2014). The cellular response to nanomaterials may go to autophagy, and the anticancer activity of C60 fullerene might be a cancer therapy because of C60 induces many characters of autophagy in cancer cells but fullerene cytotoxicity against normal cells may delay practical applications. In addition, described nanomaterials a massive group of autophagy inducer, and pointed to C_{60} fullerene caused autophagy and sensitised chemotherapeutic cell death in normal and drug resistant cancer cells dependent of ROS (Zhang et al. 2009). Although, Harhaji et al (2007), demonstrated that nano-C_{60} induced autophagy in U-251 and HeLa cell line by establishing GFP-LC3. Zhang et al (2009), nano C60 affect on GFP-LC3 protein and induced LC3-II formation

in dose-dependent and induced autophagy in the human U-251 cell line. In another hand, the Tagitinin C induced formation of autophagosome and LC3-II protein expressions, and then led to cell death in malignant U-373 cell line depend on autophagy (Lee et al. 2011). Demonstrated data showed, Beclin-1 induced by Temozolomide in human malignant glioma (Lee et al. 2015). As established, Beclin-1 has reduced in Alzheimer disease patients so strongly, in addition, in transgenic mice, Beclin-1 deficiency reduced autophagy in neuronal cells (Lee et al. 2011).

In the present study, the autophagy marker result of Beclin-1 and LC3 protein assessed by western blot in a human malignant glioma U-373 cell line in a good agreement with previous literature demonstration results. In this study, human malignant glioma U-373 cell line treated with C_{60} fullerene nanoparticle, the western blot result of Beclin-1 and LC3 protein showed, that C_{60} fullerene modulated the expression of Beclin-1 in dose-dependent. C_{60} fullerene 0.5 μM slightly but not significantly modulated the expression of Beclin-1 protein, and C_{60} fullerene 1 μM induced the expression of Beclin-1 (*$P < 0.05$) [Figure 3.5]. In another hand, the high dose of C_{60} fullerene 2 μM induced Beclin-1 protein expression two times more when compared to the control group (***$P < 0.05$) [Figure 3.5]. Recently studies supported results in this study, in human malignant glioma cells, the GFP-LC3 punctate formation and LC3-II conversion characterised the autophagic cell death (Pallichankandy et al. 2015). SNG in U-87 human malignant glioma induced autophagy through LC3-II conversion and increasing the level of Beclin-1 (Pallichankandy et al. 2015). Wang et al (2014), displayed a high concentration of ADS-I in U-373 MG, increased the autophagy by induced both of Beclin-1 and of LC3-II. Ding et al (2017), Tan IIA induced autophagic cell death in malignant glioma U-251 by up-regulating LC3-II and Beclin-1 proteins. Transformation of LC3-I to LC3-II, induced autophagy in U-373 MG (Fassina et al. 2010). Chang et al (2014), showed gefitinib-treated human malignant glioma-induced LC3 formation and conversion, may induce autophagy in human malignant glioma.

A Recent study has been approved malignant glioblastoma has resistant to apoptosis because of high expression anti-apoptotic Bcl-2 protein (Krakstad and Chekenya 2010). The apoptotic modulation effect of C_{60} fullerene in U-373 MG cells has been assessed. The significant up-regulation of apoptotic markers p53, caspase-9 and caspase-3 has been

observed after exposure to C_{60} fullerene. This study showed the expression of p53 protein significantly induced with increasing the concentration of C_{60} fullerene ($P < 0.01$) after 24 hours exposure, as shown in Figure 3.6. This result is corresponding with literature. Ershova et al and Di et al (2016), C_{60} fullerene induced the p53 protein expression. In addition, C_{60} fullerene 0.5, 1 and 2 μM considerably persuaded caspase-9 protein expression in U-373 MG cell. Caspase-3 protein expression in U-373 MG cell compared to control under C_{60} fullerene 0.5 μ inconsiderably significant induced ($*P < 0.05$) [Figure 3.6], however, C_{60} fullerene 1 and 2 μM after 24 hours treatment significantly induced [Figure 3.6]. The result in literature supported results in this thesis, for example, UV radiation leads to caspase-9 and caspase-3 mediated apoptosis in MG cell line (Hussaini et al. 2002). Titanium dioxide nanoparticles induced Caspase-3-apoptotic cell death in malignant glioma (Marquez-Ramirez et al. 2012). The results of this study strongly match to the literature, showed high expression level of p53, caspase-3, and caspase-9; high ROS and NO generation might lead to apoptotic cell death in U-373 MG cell line. Although, Zhang et al (2015), demonstrated Nano-particle C_{60} increased cellular ROS, subsequently triggers to mitochondrial pathway- apoptosis in RAW-264.7 cells. In malignant glioma U-251 cell line, herbal extract Tanshinone-IIA induced apoptosis (Ding et al. 2017). P53 protein plays a role in cell cycle arrest, DNA repair and cell death (Nakada et al. 2011). Up-regulation of p53 plays an important role in cell death (Tripathi et al. 2016). Temozolomide induced caspase-3-dependent apoptosis in human malignant glioma (Lee et al. 2015). Etoposide induced ROS generation well connected to p53 expression and the start of apoptosis in U-373 MG (Sawada et al 2001). ROS generation leads to apoptosis in MG cells (Tripathi et al. 2016). Caspase-3 induced apoptosis in Jurkat cells (Palyvoda et al. 2010). Caspase-3 leads to apoptotic cell death in MG (Fuh et al. 2009). Curcumin low concentration especially 15 and 20 leads to apoptotic cell death in U373 MG (Thani et al. 2012). However, Lee et al (2015) showed p53-dependent apoptotic cell death not appeared in U-373 MG. In addition, Hydroxylated C_{60}, which is a different form of C60, did not lead to apoptotic cell death (Yamawaki and Iwai 2006).

The member of poly (ADP-Ribose) polymerase (PARP) family PARP-1 has a critical role in DNA damage repair. However, the function of PARP has not been recognising, it binds to DNA nicks and repair the DNA breaks, PARP has been concerned in apoptosis. In another hand, in the early stages of apoptosis triggers the PARP and leading to ATP

depletion. The inactivation of PARP by caspase-3 may make easy the DNA fragmentation process (Aoufouchi et al. 1999). Fiona and colleagues (2008) have been reported the inhibition of PARP- lead to radio-sensitivity. The expression of PARP protein examined in this study after exposure the U-373 MG cell line to C_{60} fullerene. C_{60} fullerene 0.5 μM has not affected the expression of PARP protein [Figure 3.7]. However, C_{60} fullerene high dose 1 μM significantly induced PARP protein expression (***P < 0.05) [Figure 3.7], but the highest more significant expression examined after exposing to 2 μM C_{60} fullerene (***P < 0.05) [Figure 3.7]. The p21 is cell proliferation inhibitor and a cyclin-dependent kinase inhibitor. P21 frequently mutated in glioma (Manuscript 2009). By inhibiting cell cycle process, p21 can induce DNA repair and inhibit apoptosis (Abbas and Dutta 2009). The result of p21 in this study displayed C_{60} fullerene 0.5 μM did not modulate p21 protein expression considerably. The highest expression level of p21 protein showed in C_{60} fullerene 1 μM treatment in U-373 MG (***P < 0.05) [Figure 3.7]. However, the high dose of C_{60} fullerene 2 μM significantly induced the expression of p21 protein but the level of expressed p21 decreased when compared to 1 μM C_{60} fullerene treatment after 24-hour exposure to U-373 MG (**P < 0.05) [Figure 3.7]. The C_{60} fullerene induced the cell cycle arrest, activate the PARP and activate PARP cleavage in a human malignant glioma U-373 cell line after 24-hour treatment. The results were consistent with the literature, which showed Park et al (2010), displayed C60 fullerene persuade the cell cycle arrest in sub G1 and G1 phase in BAL cells. Chen et al (2009), magnolol in U-373 MG induces cell growth arrest G0/G1 phase through induction of p21 protein. While in the higher concentration could induce apoptosis. Mounira et al (2014), Limoniastrum guyonianum extract-induced apoptotic effect in U-373 MG cell line through DNA damage and PARP cleavage. Regardless of p53, Temozolomide increased G2-M phase arrest in U-373 MG (Lee et al. 2015).

Several studies demonstrated occurring autophagy and apoptosis in the same cells under some condition. Autophagy has a role in inhibiting apoptosis by damaging mitochondria and preventing the release of pro-apoptotic from mitochondria (Lee et al. 2015). In literature, some anti-tumor mediators can activate the crosstalk between apoptosis, autophagy and cell cycle arrest (Wang et al. 2014). As pointed the inhibition of autophagy increased apoptotic cell death, and suggested autophagy play a role as an anti-apoptotic task. In malignant glioma, apoptosis depends on autophagy (Lee et al. 2015).

As scientific literature showed, P53 induces autophagy in glioma (Lomonaco et al. 2009). Temozolomide induced p53-dependent autophagy in MG. Also, after the p53 knockdown, the autophagic cell death displayed in U-373 malignant glioma. In the other hand, irradiation induced autophagy by stabilising p53 in malignant glioma (Lee et al. 2015). The level of cleaved caspase-3 and cleaved PARP in U-373 MG cell treated with Celecoxib-induced caspase-dependent cell death (Sareddy et al. 2012). However, Lee with colleagues demonstrated cleaved PARP and LC3-II protein expression induced autophagy in U-373 MG and described PARP-1 (116 kDa) autophagy stimulator. Gomez-Manzano with colleagues (1997), discussed infected U251 and U373 glioblastoma with bearing p53 and p21 gene adenovirus, exposed p21 trigger apoptosis and accumulates cells in G0/G1 cell cycle, also in cells transferred p53 and p21, arrested cell cycle before going to apoptosis.

C_{60} fullerene inhibited the proliferation and induced apoptosis and autophagy in U373 cells in dose-dependent manner. Overall, our findings have suggested that water-soluble C_{60} fullerene induces cell cycle arrest, apoptosis, and autophagy in U373 cells through inducing p21/PARP, Beclin1/LC3 and activating p53/caspase-9/caspase-3 signaling pathways. Our findings shed light on the effect of C60 fullerene in glioma cancer cells.

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Curriculum Vitae

In 1986, was born in Al-Suleymaniyeh/Iraq. First, secondary and high schools graduated in Saeed Sadiq. In 2005, started college of science, department of biology in Al-Suleymaniyeh University, graduated in there in 2010. The master program started in Bingöl University at 2015. An employer in agricultural technical college of Halabja since 2011. Has a Kurdish, Arabic, English and Turkish language. Un-married.

Aryan.Faraj@gmail.com