DEVELOPMENT OF HIGH EFFICACY AND LOW TOXICITY GLIOBLASTOMA MULTIFORME (GBM) TREATMENT MODELS

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ABSTRACT

DEVELOPMENT OF HIGH EFFICACY AND LOW TOXICITY GLIOBLASTOMA MULTIFORME (GBM) TREATMENT MODELS

One of the most common types of primary brain tumors, glioblastoma (GBM), is famous for having an aggressive character because of its relatively short median overall life of only 15 months. The gold-standard drug now used in clinics, temozolomide (TMZ), is administered after radiation and surgery to remove the tumor as part of the conventional treatment for GBM. Moreover, the biological complexity of GBM genetic phenotype and resistance that develops against TMZ are the two common challenges that limit effective treatment and, therefore, hinder achieving therapeutic outcomes. Strong interest was raised in pharmacological ascorbate based on the recent clinical research studies that reported its role in improving quality of life and extending survival in a variety of cancer types. Likewise, boron compounds are also being increasingly recognized for its anti-cancer properties. In the current study, the anti-cancer effect of Vitamin-C, sodium pentaborate pentahydrate, and their combination are addressed in the *in vitro* GBM models of IDH-MUT-U87 and U373MG cell lines. These compounds were examined in terms of understanding their effects on various cellular processes, including apoptosis, autophagy, cell migration, cell cycle, and DNA Damage Response (DDR). Furthermore, in the second part of the study, a combination of Olaparib and Vitamin-C was identified to exert a synergistic effect in cell toxicity measurements. Strikingly, we also observed that the combination of Vitamin-C and NaB promoted an anti-tumorigenic effect through activation of DDR signaling, induction of autophagy, and apoptosis mechanism in the IDH-MUT-U87 cell line. Finally, various combinations of these compounds exhibited anti-cancer effect via DDR and ROS-dependent signaling in the U373MG cell line. In this study, the anti-cancer effect of the combination of NaB and Vitamin-C in GBM cancer models was highlighted. The results of this study may offer an insight into the potential use of the combination of NaB and Vitamin-C as a drug to treat cancer.

ÖZET

YÜKSEK VERİM VE DÜŞÜK TOKSİSİTELİ GLİOBLASTOMA MULTİFORME (GBM) TEDAVİ MODELLERİNİN GELİŞTİRİLMESİ

Glioblastoma (GBM), en çok karşılaşılan primer beyin tümörlerinden biridir ve ortalama sağ kalım süresinin 15 ay gibi son derece kısa olmasından kaynaklanan agresif fenotipiyle bilinir. Standart GBM tedavisi tümör cerrahisini takip eden radyasyon ve klinikte kullanılan altın standart olan Temozolomid (TMZ) tedavilerini içerir. Ayrıca, GBM genetik fenotipinin biyolojik karmaşıklığı ve TMZ'ye karşı gelişen direnç, etkili tedaviyi sınırlayan ve dolayısıyla terapötik sonuçlara ulaşılmasını engelleyen iki yaygın zorluktur. Çeşitli kanser türlerinde yaşam kalitesini iyileştirme ve sağkalımı uzatmadaki rolünü bildiren son klinik araştırma çalışmalarına dayalı olarak farmakolojik askorbata büyük ilgi gösterildi. Benzer şekilde, bor bileşikleri da kanser önleyici özellikleriyle giderek daha fazla tanınmaktadır. Mevcut çalışmada, IDH-MUT-U87 ve U373MG hücre hatlarının *in vitro* GBM modellerinde Vitamin-C, sodyum pentaborat pentahidrat (NaB) ve bunların kombinasyonunun anti-kanser etkisi incelenmiştir. Bu bileşikler, apoptoz, otofaji, hücre göçü, hücre döngüsü ve DNA Hasar Tepkisi (DDR) dahil olmak üzere çeşitli hücresel süreçler üzerindeki etkilerini araştırılmıştır. Ayrıca, çalışmanın ikinci bölümünde Olaparib ve Vitamin-C kombinasyonunun hücre toksisitesi ölçümlerinde sinerjistik bir etki gösterdiği belirlendi. Çarpıcı bir şekilde, Vitamin-C ve NaB kombinasyonunun, IDH-MUT-U87 hücre hattında DDR sinyalinin aktivasyonu, otofajinin indüklenmesi ve apoptoz mekanizması yoluyla bir anti-tümorijenik etkiyi desteklediğini de gözlemledik. Son olarak, bu bileşiklerin çeşitli kombinasyonları, U373MG hücre hattında DDR ve ROS'a bağlı sinyalleşme yoluyla bir anti-tümorijenik etkiyi desteklediği gözlenmiştir. Son olarak, bu bileşiklerin çeşitli kombinasyonları, U373MG hücre hattında DDR ve ROS'a bağlı sinyalleşme yoluyla anti-kanser etkisi sergilediği belirlenmiştir. Bu çalışmada GBM kanser modellerinde NaB ve Vitamin-C kombinasyonunun anti-kanser etkisi vurgulanmıştır. Bu çalışmanın sonuçları, NaB ve Vitamin-C kombinasyonunun kanseri tedavi etmek için bir ilaç olarak potansiyel kullanımına ilişkin bir tahmin sunabilir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

3D-CRT	Three-dimensional conformal radiation therapy
AA	Ascorbic acid
AG-221	Enasidenib
AG-881	Vorasidenib
ATCC	American type culture collection
ATG5	Autophagy related 5
ATR	Ataxia telangiectasia and rad3 related
ATRX	Alpha-thalassemia mental retardation X-linked
ATP5B	ATP synthase subunit beta
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BER	Base excision repair
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
CAK	CDK activating kinase
CDK1	Cyclin dependent kinase 1
CDK2	Cyclin dependent kinase 2
CDK4	Cyclin dependent kinase 4
CDK6	Cyclin dependent kinase 6
CDKN1A	Cyclin-dependent kinase inhibitor 1A
CMV	Cytomegalovirus
CT	Computed tomography

D-2-HG	D-2-hydroxyglutarate
DAPI	4′,6-diamidino-2-phenylindole
DC	Dendritic cells
DDR	DNA damage response
DHA	Dehydroascorbic acid
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double strand DNA break
EBV	Epstein-barr virus
ECs	Endothelial cells
EDU	Ethynyl-2′-deoxyuridine assay
EGFR	Epidermal growth factor receptor
FADD	Fas-associated death domain
FBS	Fetal bovine serum
FDA	Food and drug administration
GBM	Glioblastoma multiforme
GSH	Glutathione
GULO	L-gulono-lactone oxidase
H_2O_2	Hydrogen peroxide
HIF1-α	Hypoxia-inducible factor 1-alpha
ICC	Immunocytochemistry
IDH	Isocitrate dehydrogenase

IDH1 mutant-U87	Glioma IDH1R132H mutant isogenic cell lines
LC3A	Microtubule-associated protein 1A/1B light chain 3A
LC3B	Microtubule-associated proteins 1A/1B light chain 3B
MGMT	O6-methylguanine-DNA methyltransferase
MMR	DNA mismatch repair
MRI	Magnetic resonance imaging
NaB	Sodium pentaborate pentahydrate
NF-kB	Nuclear factor kappa B
NHA	Normal human astrocyte cell line
NK	Natural killer
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
OS	Overall survival
PARP	Poly-ADP ribose polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PDL-1	Programmed death-ligand 1
PI	Propodium iodide
PTEN	Suppressor gene of phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
RB	Retinoblastoma protein
ROS	Reactive oxygen species
TET	Ten eleven translocation

TMZ	Temozolomide
TNFR1	Tumor necrosis factor receptor 1
Tp53	Transcription factor
TRADD	TNFR1-associated death domain protein
U-373MG	Human glioma cell lines
VEGF	Endothelial growth factor
Vit-C	Vitamin-C

1. INTRODUCTION

1.1. CANCER

Cancer is a condition characterized by aberrant cell division, the expansion of damaged cells that may spread to other bodily regions, and is brought on by genetic or epigenetic alterations in somatic cells. [1]. In other words, cancer can also be defined as a group of neoplasms that form as a result of dysregulated tissue growth. The main distinction between cancer and normal cells is that normal cells mature, live a consistent amount of time, and eventually die from aging or injury. Douglas Hanahan and Robert Weinberg presented the "Hallmarks of cancer" and described crucial biological processes that contribute to tumor proliferation in their important review (Figure 1.1) [2–4]. 11 years later these authors added other emerging processes that are implicated in tumor growth in an update of their review, describing reprogramming of energy metabolism and evasion of immune response and immune checkpoint, as emerging culprits in both onset and maintenance of tissue transformation [3]. Below we summarize each of these pro-tumorigenic processes and how they contribute to transformation highlighting the key factors involved.

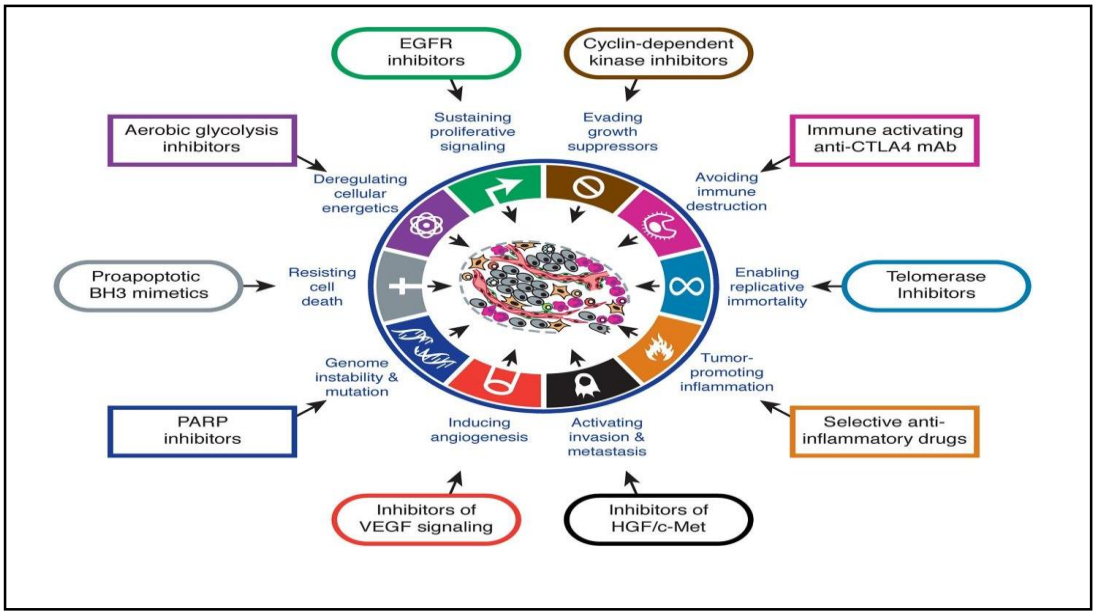

Figure 1.1. Schema of the hallmark of cancer [3].

In healthy tissue homeostasis, cell proliferation, and cell death signals take place at an equilibrium. Also, in healthy tissue, growth factors regulate signalling proteins and cell proliferation [5]. Cancer cells continuously activate growth and division pathways even in the absence of normal growth signals. Moreover, cancer cells have the capability to maintain their own proliferation and to grow uncontrollably. This might be a result of the mutations in those genes related in the growth regulatory pathways such as (Epidermal growth factor/their receptor EGR/EGFR). Often times these mutations confer gain-of-function in these growth-related gene products, which become perpetually activated, and hence account for the neoplastic tissue outgrowth.

Genes that act to inhibit cell proliferation and tumor growth are called tumor suppressor genes. These genes are frequently deleted or inactivated in tumors, an event that also contributes to the abnormal growth of cancer cells [6]. There are two main tumor suppressors genes such as the transcription factor (Tp53) and the retinoblastoma protein (RB) that regulate cellular responses to genotoxic stress [7]. These proteins are essential for controlling the cell cycle and induction of apoptosis. Because tumor suppressor genes are often inactivated in cancer cells, they can escape growth control checkpoints and, therefore, can continue grow uncontrollably.

Apoptosis is known as a programmed cell death that is controlled by several physiological and biochemical processes. It is a controlled form of cell elimination, which is essential for the maintenance of genomic integrity, embryonic development, elimination of deficient tissue, homeostasis, and change in morphology [8,9]. The apoptosis process is also as a natural defense or barrier against cancer cell. There are two significant apoptotic mechanisms, including the extrinsic and the intrinsic pathways. In the death receptor-dependent pathway, binding of a death receptor (TNFR1 or Fas) by their ligands (TNF and FAS ligands) initiates the programmed cell death events [10]. The intracellular death domain of death receptors binds adaptor proteins like receptor-associated death domain (TRADD) and Fas-associated death domain (FADD) [11]. When these proteins bind to death receptors, a ligand-receptor-adapter protein complex forms promoting the assembly of the death-inducing signaling complex (DISC), a key downstream complex involved in the execution of apoptosis [12]. Next, DISC activates caspase 8, which is known as the initiator of apoptotic cellular clearance mechanisms [13]. Conversely, the mitochondrial pathway causes programmed cell death by allowing the permeabilization of the mitochondrial

membrane and encouraging the release of cytochrome c into the cytoplasm. [14]. BCL-2 protein is a key factor of the intrinsic pathway that executes an important role in cell survival-related processes. Mainly, proteins that are related to BCL-2 protein constitute a family of factors known as the BCL-2 family that is divided into two main groups. In the first group, factors such as Bax, Bad, Bak, Bid, Bcl-Xs, Bik, Bim, and Hrk are recognized for their pro-apoptotic roles, while the proteins of the second group such as Bcl-2, Bcl-W, Bcl-XL, Bfl-1, and Mcl-1 are known for their anti-apoptotic role [15]. Pro-apoptotic proteins operate by triggering the release of cytochrome-c from the mitochondria, whereas anti-apoptotic proteins control apoptosis by inhibiting this release. The formation of a distinct complex named as Apoptosome and the activation of caspases 8 and 9 are triggered by the release of cytochrome-c from the mitochondria through the intrinsic route. Cell death is brought on by these activated caspases through activation of downstream executor caspases like caspase 3.

Replicative immortality becomes possible by telomeres, which are preservative formation that "cap" the ends of chromosomes and, thereby prevent chromosomal end erosion and maintain genome integrity [16]. Because of and inherent replication problem in each replicative cycle and stress, telomeres become progressively shortened in each cell division cycle in such a way that they ultimately become ineffective [16]. Cancer cells are able to circumvent telomere shortening through activation of telomerase which are the key enzymatic activity for the synthesis of telomeric repeats.

The phenomenon of angiogenesis is when the endothelial cells that line the blood vessels proliferate to generate new blood vessels [17]. Also, in healthy tissues, angiogenesis can be regulated by the synthesis of pro- and anti-angiogenic factors [18]. This balance is often perturbed in initiating tumors in favor of excessive insufficient angiogenesis providing the resident tumor cells with oxygen and nutrients propelling their growth [19]. Angiogenesis process, which is controlled by numerous anti-angiogenic and pro-angiogenic factors, also involves the breakdown of the basement membrane as well as stimulation, proliferation, and migration of endothelial cells (ECs) [20]. Under normal conditions, angiogenesis is closely regulated by a balance of stimulators and inhibitors. For example, the fully understood example of Vascular endothelial growth factor (VEGF) is a particular and effective promoter of angiogenesis [21]. VEGF is a cytokine that acts on vascular endothelial cells to promote the formation of new blood vessels, and it is essential for both healthy and malignant angiogenesis [22]. Additionally, an organization of blood vessels that feed and remove waste

from a tumor is activated, turning a confined mass of tumor cells into an invasive and metastatic mass. Invasion occurs when cancer cells immediately infiltrate and extend into the tissues that are close by. Furthermore, separation of the tumor cells from their original site, migration to distal sites to initiate secondary lesions at these loci is referred to as metastasis. Metastasis is a key process that limit prognosis of clinical disease. At the molecular level, loss of e-cadherin is a characteristic event that promotes invasion and metastasis by cancer cells. E-cadherin facilitates the formation of epithelial lining of the organs by forming adherent junctions in-between the neighboring epithelial cells and warranting the immobility of cells within the layers [23].

Genomic instability is defined as a sustained generation of the chromosomal alterations and genomic changes in the nucleic acid sequences in cancer cells [24]. Also, this process causes an accumulation of genetic changes that provide tumor cells with survival and proliferative advantages [25]. Furthermore, genomic instability provides the cancer cells with the alterations they need to survive and metastasize. It also, performs a significant role in the development of cancer by evading immunological regulation, achieving unregulated cell proliferation, and increasing DNA damage [26]. In addition, inflammatory processes can be controlled by cancer cells in a way to aid their growth and survival. Also, cell of immune complete their assigned mission of engulfing and eliminating foreign invaders throughout a typical immune response. In most cases, immune cells have been damaged by cancer cells in the tumor microenvironment. As a consequence of the transformation, resident immune cells that normally should display an anti-tumor effect, can be found to exert pro-tumorigenic properties enhancing the growth and spread of tumor cells. These immune cells release substances that are pro-survival and pro-migratory, For example, NF-Kb (Nuclear Factor kappa B), inflammasome signaling, and immune checkpoint signaling are pivotal molecules and signaling pathways in modulation of the immune response in the tumor microenvironment [27] In the sense that an environment that promotes malignancy, tumor development, and invasion becomes created when cancer cells interact through the NF-B signaling pathway [28].

Another distinctive feature between healthy and cancer cells is the altered programming of energy metabolism in the latter [29]. Metabolic reprogramming is essential for carcinogenesis, cancer growth, and tumor responses to medication because it gives cancer cells the capacity to respond to stress and the surrounding environment [30]. Several

different factors such as oncogenes, hypoxia-induced factors, growth factors, and malfunctioning tumor suppressors are involved in the metabolic reprogramming of the cancer cells [31]. These modifications affect cellular metabolism, particularly glucose absorption, which is markedly increased in cancer [32].

The defense mechanism of organisms that responds to external threats is the immune system, which is crucial in the formation and development of cancer. [33]. Generally, the immune system scans for damaged cells and eliminates them before they can become carcinomas and tumor cells. In this process, specialized immune cells can identify and eliminate cancer cells, such as T cells, dendritic cells (DC), natural killer (NK) cells and neutrophils [33]. Dendritic cells and macrophages related to tumors can stimulate the proliferation of cancer cells through producing cytokines and chemokines that encourage angiogenesis and suppress T cell function. Similarly, Treg cells, can reduce the immunological response to cancer by preventing T cells from infiltrating and expanding in the tumor bed [34]. Fundamentally, the immune system cannot totally eliminate the cancer cells since they are not permitted to grow uncontrolled.

1.2. GLIOBLASTOMA MULTIFORME (GBM)

Glioblastoma multiforme (GBM, WHO grade IV astrocytoma) is the most fatal and aggressive kind of malignant primary brain tumours, constituting almost 48.6 % of these tumours [35–38]. GBM continues to have a patient survival rate of 10% five years after diagnosis and an average survival duration of 15 months [39]. GBM is derived from oligodendrocytes and primary astrocytes [40]. The frontal, temporal, parietal, and occipital lobes of the supratentorial brain contain a significant number of glioblastoma tumours, but the cerebellum, brain stem, and spinal cord rarely generate them [41]. Although glioblastoma is less common than other cancers like lung, breast, prostate, and colon cancer, it is frequently more deadly and life-threatening than these other tumor types. Therefore, despite of maintaining a lower frequency than other cancer types including breast, colon, prostate, and lung cancer, glioblastoma frequently has a higher mortality rate than these other tumour types.

1.2.1. EPIDEMIOLOGY OF GLIOBLASTOMA MULTIFORME

The incidence of gliomas varies according to age, sex, race, and geographical region. Despite of having an incidence of less than 10 in 100,000 people worldwide, GBM is a rare tumor with a poor prognosis and stills presents as a serious public health risk [42]. Although GBM can occur at any age, the highest occurrence is seen in over the age of 65 [43] and also, it is rarely found in children [39]. Men (5.51 /100.000 population) are estimated to have an increased risk of GBM than women (3.65 /100.000 population) [39,44].

Furthermore, Asians and Native Americans have a lower frequency of the GBM than the white people, who can receive this diagnosis twice as often as black and African-American people [45].

No definite association with GBM has been found with smoking, environmental exposures, use of cell phones, and nutrition [40]. A high risk of GBM has been reported in the individuals who have rare syndromes such as Li-Fraumeni syndrome, neurofibromatosis I, and Turcot syndrome [46,47]. As sitomegalovirüs (CMV) and Epstein-Barr virus (EBV) have been detected in glioma samples, these two viruses have been associated with GBM, but the reason for this is currently unknown [48,49].

1.2.2. ISOCITRATE DEHYDROGENASE (IDH) MUTATION OF GBM

There are three different isoforms of the important enzyme isocitrate dehydrogenase (IDH), which plays a role in crucial metabolic reactions of the Krebs cycle, lipogenesis, and glutamine metabolism, and redox balance [50,51]. Also, there are three subtypes IDH such as cytosolic $NADP^+$ dependent (IDH1), mitochondrial $NADP^+$ dependent (IDH2) and mitochondrial NAD^+ dependent (IDH3) [52]. Moreover, IDH3 plays a crucial function in energy generation and has its own unique sequence although IDH1/2 have similar sequence (%70) and catalyze reversible process [53].

It has been demonstrated that the IDH1 mutations in cancer produce 2-hydroxyglutarate due to the neomorphic catalytic activity of this mutant IDH 1/2 enzyme that transforms alpha-ketoglutarate into 2-hydroxyglutarate that inhibits the histone lysine demethylases. [44]. While IDH3 enzyme mutations alters the properties of the enzyme to act as an electron

acceptor [52]. IDH1 mutations are mostly found in the hotspot arginine at codon R132 substituting this Arginine into various other amino acids, including R132S, R132G, R132H, and R132C [54]. Likewise, similar mutations are reported for the codon 172 of IDH2 [54].

By generating NADPH, the IDH1/2 enzymes provide an adequate amount of reduced glutathione (GSH) and peroxiredoxin. This safe-guards the redox equilibrium and defends the cell against oxidative damage generated by several types of cellular stresses [55]. IDH1 produces NADPH, which is important in lipid metabolism due to its resistive role against reactive oxygen species (ROS) generated upon lipid oxidation [56]. By producing chemicals like NADPH and alpha ketogluyarate, which have potent reductive capabilities and provides defense against DNA damage upon their associations with glutathione and thioredoxin, IDH1/2 contributes significantly1 to the defense against oxidative stress [57]. More than as 65% of the NADPH in the human brain is produced by the IDH1-driven process, making it the largest manufacturer of NADPH in the brain [58]. Additionally, also IDH1/2 plays a critical role in the glutamine metabolism under hypoxic conditions and changes to the electron transport chain [59].

There are several therapeutic strategies tested including direct targeting of IDH mutations, targeting DNA repair mechanism, and immunotherapies for IDH mutant-driven glioma.

Direct targeting of the IDH mutant enzyme is an intensively used strategy. In one study, AGI-5198, the first synthetic inhibitor of the IDH mutant, reported to block D-2-HG production and reduce xenograft growth in the in-vivo experiments [60]. In addition, ivosidenib (AG-120) for IDH1 and vorasidenib (AG-881) for IDH1/2, and enasidenib (AG-221) for IDH2 are those IDH-mutant inhibitors that were approved by the Food and Drug Administration (FDA) as a treatment for GBM cases [61–63]. Despite, IDH-mutant inhibitors were effective in reducing 2-HG production, they remained ineffective in reversing the histone hypermethylation phenotype and failed to significantly slow the proliferation of glioma cells according to some studies [61,64].

Multiple research investigations have demonstrated that Temozolomide (TMZ), irradiation, and their combination treatment are effective in treatment IDH-mutant gliomas [65,66]. Also, the integrity of double strand DNA break (DSB) repair is damaged in tumors with IDH mutations accompanied with increased generation of D-2-HG [67,68].

When IDH mutant cells are exposed to genotoxic therapies like radiation or chemotherapy, it involves DNA repair pathway to ensure survival and genomic integrity [69]. The PARP inhibitor olaparib can lead to lethality by depriving them of this crucial DNA repair process [70].

Potential immunotherapy targets include IDH mutations. Moreover, the fact that programmed cell death-1 (PD-1) and its ligand (PDL-1) are silenced in tumors caused by D-2-HG compared to IDH wild-type gliomas further suggests a higher level of T-cell activation [71]. Other effective treatments that prevent tumor cells from escaping the immune system include avelumab, an immune checkpoint inhibitor [62]. These primarily function by preventing the association between immune checkpoint ligands and cytotoxic T cells and eliminate the inhibitory effects of the ligands on lymphocytes.

1.2.3. MOLECULAR BIOLOGY OF GBM

Molecular biomarkers have become more significant in the pathologic classification and treatment of glioblastomas, and also in the evaluation of its prognosis and therapeutic response. O6-methylguanine-DNA methyltransferase (MGMT) is one of the biomarkers found on chromosome 10q26 that produces a protein involved in DNA repair. Epigenetic silencing of MGMT by methylation of its promoter has been found to be a good prognostic indicator and to be associated with an improved overall survival (OS) in patients following radiation therapy and chemotherapy (temozolomide) [62,72].

Another biomarker is (alpha-thalassemia mental retardation X-linked (ATRX) that is located on chromosome Xq21.1 Furthermore, coexistence of ATRX mutations with IDH1 and TP53 mutations in GBM cells is considered as a positive prognostic factor [73].

In addition, the telomerase reverse transcriptase (TERT) enzyme, responsible for telomere synthesis in the 3' end in replication, is found to be hyperinduced in GBM tumors. The TERT gene promoter alterations, which are frequently found in GBM patients, result in increased telomerase activity, indicating that telomere maintenance is essential for brain tumors [74].

A functional form of homotetrameric tumor suppressor protein p53 is important for regulating the integrity of the genome, proliferation, and other cellular processes [75]. The mutation of p53 function leads to its changed expression and this promotes malignant propensity of these cells through enhancement of their invasive, metastatic, proliferative and genomic instability properties [76,77].

Suppressor gene of Phosphatase and tensin homolog (PTEN) is located on chromosome 10q23. A poor prognosis for GBM is attributed to loss of PTEN expression carried out by the loss of heterozygosity. The majority of PTEN, a protein displaying both protein phosphatase and lipid phosphatase activities, act as a lipid phosphatase [78]. Moreover, Absence of PTEN affects the regulation of cell proliferation, growth, and survival because PTEN suppresses the PI3K/Akt pathway. [79].

1.2.4. GBM TREATMENT

The current standard of care for newly diagnosed GBM cases involves surgical resection, chemotherapy, and radiotherapy [80]. In addition, the inclusion of temozolomide (TMZ) to the therapy regimen indicates one of the most significant recent developments in therapeutic approaches [73]. Furthermore, comprehensive and complete surgical excision of GBM is difficult due to the fact that these tumors are frequently invasive and frequently localize to the regions of the brain that control speech and motor function. Additionally, because GBM tumors are extremely invasive and infiltrating tumor cells invariably remain in the surrounding brain, the disease will either worsen or recur if the original tumor mass is not completely removed. [81].

After the introduction of the "brain mapping" technique, which uses cerebral cortical stimulation to help localize the cortex region of the brain that could be protected in brain surgery, the technology allowed a simpler method to remove brain tumors. Computed tomography (CT) and magnetic resonance imaging (MRI) techniques are the most commonly used techniques used to determine tumor margins and collect biopsies more precisely. Furthermore, because to its superior soft tissue contrast and excellent resolution, computed tomography (CT) is the gold standard in brain tumor surgery. This makes it more trustworthy than magnetic resonance imaging (MRI), which is a comparatively more established imaging technology but was once more frequently utilized. Due to high

resolution of CT, the blood arteries, tumor's margins, inside the tumor, necrotic sections, blood perfusion, and bleeding, can all be clearly visualized [82,83]. Another technique of high resolution imaging for gliomas is fluorescence imaging that does not penetrate the blood brain barrier and it exclusively enters high permeability areas with glioma cancer [84].

Temozolomide is the widely used chemotherapy agent used for the treatment of GBM post-surgery and works by methylating the purine bases to cause genotoxic damage to cancerous cells [85]. TMZ triggers autophagy, apoptosis, and cellular senescence in tumor cells. Particularly, when it is used together radiation, the drug's radiation-sensitizing effects potentiate the radiation-induced cancer cell death [85,86]. In addition, there are different alkylating agents such as lomustine, fotemustine and both drugs are utilized orally in the treatment of recurring glioblastoma owing to their strong lipophilicity and small size [87,88]. Furthermore, the chemotherapy drug bevacizumab is used to treat recurrent GBM [80] and could potentially target vascular endothelial growth factor A (VEGF-A)-dependent angiogenesis.

Protons, gamma photons, and X-ray photons are the new forms of radiation that are able to target tumors with higher precision enabling removal tumor cells that are too small to be removed by surgery. Also, three-dimensional conformal radiation therapy (3D-CRT) is used for small or microscopic tumor that cannot be removed by surgery. CT scans and MRI imaging, X-rays are applied to target tumor cells from various angles. Another radiation technique of conformal RT, which has fewer adverse effect and targets just cancer cells, having no impact on the normal cells, is an often used approach for treating remaining GBM cells [89].

1.3. ASCORBIC ACID

Ascorbic acid (AA) often known as vitamin C is an antioxidant, water soluble molecule and enzyme cofactor derived from glucose found in all plants and animals [90]. Since, enzyme L-gulono-lactone oxidase (GULO), which catalyzes the ascorbic acid last stage production, is not expressed in human, this co-enzyme must be obtained from outside resources [91].

Ascorbic acid (AA) can be found in multiple redox forms depending on the biological circumstances as a seen Figure 1.3. Also, both intracellularly and extracellularly Vitamin-C a completely reduce and capable of being oxidized. Reactive oxygen species (ROS) oxidize extracellular ascorbate, creating the weak radical intermediate ascorbate radical ($Asc^{\bullet -}$), that is subsequently completely oxidized producing dehydroascorbic acid (DHA) [92]. Only 1-5% of vitamin C is found in the human body as DHA, which has a short half-life, is either transported within cells (Figure 1.3.) and irreversibly hydrolyzed into 2,3-L-diketoglutonate (2,3-DKG). The subsequent breakdown of 2,3-DKG into oxalic acid and threonic acid causes a net loss of vitamin C [93].

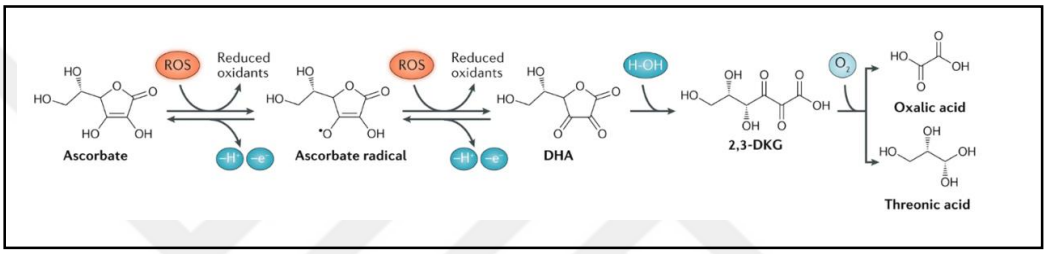

Figure 1.2. Ascorbic acid degradation [94].

The central nervous system's a state of equilibrium and optimal function depend on Vitamin-C. The brain uses a significant amount of oxygen (20%) and glucose (25%), indicating rapid digestion and higher levels of free radical generation. In addition, the biologically most prevalent antioxidant found in brain tissue is Vitamin-C [91]. Moreover, total average Vitamin-C concentration of brain lower than liver, lens, and adrenal gland. The normal levels of Vitamin-C in brain is essential for carrying out a variety of CNS functions, including the synthesis of catecholamine, the formation of myelin, the enhancement of synaptic activity, and the control of precursor cell proliferation and differentiation [95–97].

It has been demonstrated that ascorbate or high vitamin C concentrations have pro-oxidative actions that harm cancer cells [98–100]. Also, Vitamin-C functions as a pro-oxidant at concentrations more than 1 mM in plasma, according to a research study [101]. This happens when transition metals such as copper and iron are present and are reduced by Vitamin-C before reacting with hydrogen peroxide (H_2O_2). It stimulates the production of extremely powerful hydroxyl radicals that can harm or kill the tumor cell (Figure 1.3). However, in the situation of normal cells, H_2O_2 is instantly eliminated since both catalase and glutathione peroxidase (GP) are present in high concentrations in the blood stream. As long as they have enough, it produces hydrogen peroxide (H2O2), which is particularly harmful to cancer cells.

Because of the poor blood perfusion in tumor cells, the antioxidant enzyme concentration is significantly low, and H_2O_2 continues to harm the cells [102–104]. In addition, antitumor effects of Vitamin-C can lead to induction of apoptotic pathways, increased pro-oxidant-dependent damage to cancer that could not be repaired, and unstable metabolite formation from ascorbate in plasma at high levels that is very toxic for the tumor cells [105,106].

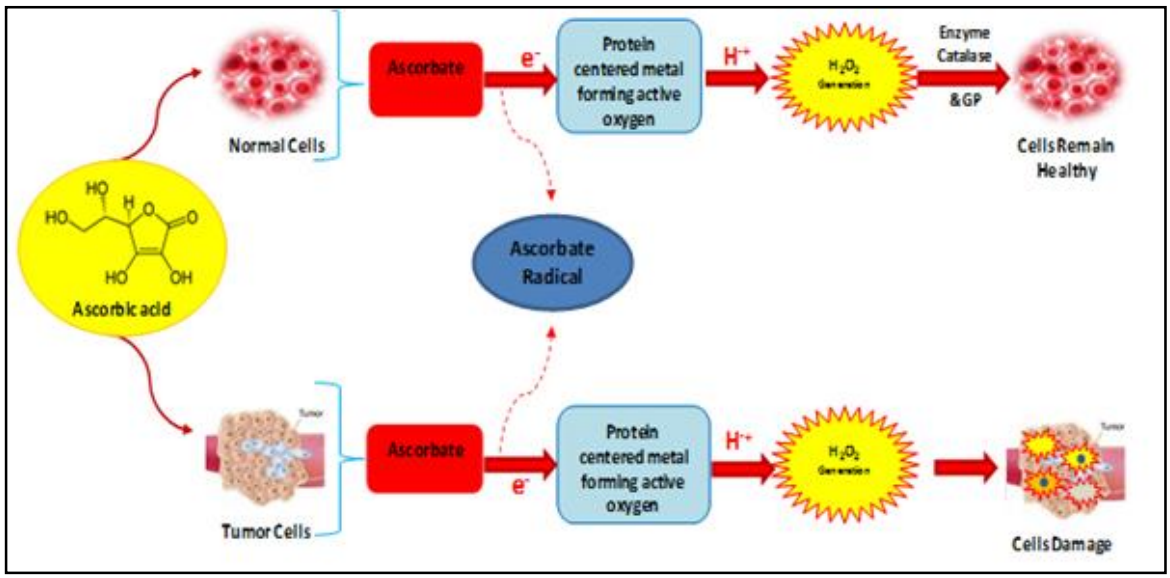

Figure 1.3. Ascorbic acid or Vitamin-C anti-cancer effect [107].

The most well-known mechanism of ascorbic acid's anticancer action in different carcinomas is the ROS-mediated mechanism. High ascorbic acid concentrations given systemically function in vivo as a pro-oxidant, generating ROS by the Fenton reaction [108]. A potent ROS derivative, H_2O_2, which is created cell outside, instantly diffuses within the cell and takes [109]up antioxidants such as decreased glutathione and NADPH [110]. In addition, DNA damage elevates PARP activity, which decreases NAD^+ levels and restricts glycolytic system operations. ROS formation promotes oxidative stress and DNA damage [111].

HIF1 could prove to be an advanced cancer treatment method since it contributes in angiogenesis and the control of the glycolytic system, that are the two essential mechanisms for the growth and progression of cancer. Vitamin C deficiency impairs HIF hydroxylase activity while increasing HIF1-dependent transcriptional activity promoting angiogenesis [112,113]. Therefore, Vitamin-C coenzyme activation may limit HIF expression and activity in tumor and suppress cancer cell proliferation by blocking angiogenesis.

Vitamin-C plays a role in cancer epigenetics and it could be activated cofactor of Ten Eleven Translocation (TET) enzymes involved in DNA demethylation [114]. For TET activation, Vitamin-C might donate an electron to Fe^{3+} to generate reduceed Fe^{2+} [93]. In addition, stem cell reprogramming and maintenance of self-renewal are both mediated by TET activity and intracellular vitamin C [115,116]. In cells with IDH mutations, isocitrate dehydrogenases (IDH) mutations decrease alpha ketoglutarate, a substrate for TETs and jumonji-domain histone demethylases (JHDMs), and enhance DNA methylation, controlling gene expression that results in carcinogenesis, including glioma. [117]. Vitamin-C is required for JHDMs to function properly and may suppress oncogenic gene expression by increasing histone demethylation [118]. Basically, Vitamin-C improves the hypermethylation condition associated with malignancies by TETs and JHDMs and modifies gene expression to have anticancer effects.

1.4. BORON

Boron is a naturally occurring metalloid that is brittle, black, and glossy in crystal form. Since 1923, boron has become acknowledged as a crucial component for plants, having important ramifications for plant physiology. Along with plants, humans too contain boron in a variety of tissues. In sufficient concentrations, borates or boric acid make up the element in healthy tissues. The body has between 3 and 20 milligrams of boron. Boron compounds are found in varying concentrations in different tissues [119]. A Low-Boron diet has been linked to a range of general [120] health issues and elevated cancer risk. Arthritis, soft cartilage illness, memory loss, osteoporosis, hormone disorders, and a decline in libido are the most typical signs of boron deficiency. The amount of boron consumed daily varies based on the type of food consumed, the use of particular personal care products, and the water content of the boron. Uncertainty still exists over the precise amounts of boron that the human body needs; determining these amounts will necessitate further research on the biological uses of boron and the control of boron exchange. Boron compounds have been found to have anti-carcinogenic properties in an increasing number of studies. There is a correlation between boron-rich diets and areas with low rates of numerous malignancies, including prostate, breast, cervical, and lung cancers. A large range of Boron based compounds can be produced and studied for chemoprevention and chemotherapy since Boron has a modest atomic mass and its chemistry contains neutrophilic and electrophilic

reactivity. Studies have shown that eating a diet high in dietary boron lowers the risk of lung cancer in women who smoke, as well as the risk of prostate and cervical cancer. Naturally existing and synthesized chemicals containing boron have become more popular in recent years as anti-cancer agents, particularly for tumors that are incurable or have a high aggressiveness. By inhibiting serine proteases, NAD dehydrogenases, modulating mRNA splicing, cell division, receptor binding mimicking, and inducing apoptosis, chemicals containing borax affect the physiology and reproduction of cancer cells [121]. When using Boron chemistry to combat cancer cells, there are two distinct methodologies: chemotherapy and diet-based chemoprevention. Boron supplemented diets and the incidence of several cancer types were found to be negatively correlated. The inhibition of numerous enzymatic processes, including serine proteases, mRNA splicing, DNA replication, NAD-dehydrogenases, thymidilate synthesis, non-histone chromatin methylation, DNAses, RNAses, cathepsin, and others are amongst the potential mechanisms underlying the anti-cancer activity of Boron-based compounds. Chemicals containing borax and $Ca2^{+}$ receptors, prevent cell division, imitate nuclear receptor binding, and trigger apoptosis [122].

2. MATERIALS AND METHODS

2.1. CELL LINES AND CELL CULTURE CONDITIONS

IDH1 mutant-U87 Isogenic Cell Line (HTB-14, glioma IDH1R132H mutant isogenic cell lines), U-373MG (HTB-17, human glioblastoma cell lines), NHA (normal human astrocyte cell line) were originally purchased from American Type Culture Collection (ATCC, Rockville, MD). IDH1 mutant U87 cell lines were cultured in Eagle's minimum essential medium (EMEM, #30-2003). The U-373MG cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM High Glucose, #41966-029, Invitrogen, Gibco, UK). NHA cell lines were cultured in Astrocyte Growth Medium BulletKit (AGM, #CC-3187). EMEM and DMEM High Glucose medium were supplemented with 1% Penicillin/Streptomycin/Amphotericin (PSA, Invitrogen, Gibco, UK) and 10% fetal bovine serum (FBS, #10500-064, Invitrogen, Gibco, UK). AGM Medium contained SingleQuotsTM Supplements (CC-4123) for growth astrocyte cell lines. Furthermore, each cell is maintained at 37 °C and 5% CO2 in a humidified incubator.

2.2. CYTOTOXICITY ASSAY

IDH1 mutant-U87 cells, U-373MG cells, and NHA cells were cultured in 96-well plates at a density of 2250 cells/well, 2500 cells/well and 5000 cells/well respectively. The subsequent day, each cells was treated with alone TMZ, Olaparip, NaB. Futhermore, double combination of treatment Vitamin-C doses were selected the doses of cell viable (non-toxic doses) for IDH1 mutant-U87 cells and U-373MG cells while the highest viable or applicable dose (500 μg/ml) in the cancer cell was selected for normal cell line. Cell viability was measured using the MTS assay (3-(4,5-dimethyl-thiazol-2)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolim salt (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) according to manufacturer’s instructions for 24, 48,72 hours. After aspirated the treatment containing medium, an MTS solution (a PBS containing 4.5 g/L D-glucose and 10% MTS) was added and incubated 60 minutes at 37 °C. Then, their absorbance was measured at 490nm by using an ELISA plate reader (Biotek, Winooski, VT).

GraphPad Prism software was used to determine IC-50 values and all values were shown in table 2.

2.3. ANNEXIN V ASSAY

IDH-MUT-U87 and U373MG cells were seeded into T25 flasks at a density of 75 $x10^4$ and $10x10^5$ respectively. After day, cells were treated for NaB, Vitamin-C and their combination further incubated for 72h at 37°C. After 3 days, they were harvested and washed with PBS. The manufacturer's instructions for the Annexin V test (#sc-4252AK, Santacruz Biotechnology, USA) were followed. After, it was resuspended with Annexin V binding buffer and divided into four groups (Annexin V, propidium iodide (PI), Annexin V + PI, and NC). Data were analyzed by using FACSCalibur (BD biosciences) flow cytometry device.

2.4. CELL CYCLE ANALYSIS

IDH-MUT-U87 and U373MG cells were seeded into T25 flasks at a density of 75 $x10^4$ and $10x10^5$ respectively. After day, NaB, vitamin C, and their combination were applied to the cells, and they were then incubated for a further 72 hours at 37°C. They were collected after three days, rinsed with PBS, and fixed for at least two hours in 70% ice-cold ethanol at -20°C. After being permeabilized with 0.1% Triton-X-100, cell pellets were treated for 30 minutes at room temperature with 20 g/ml RNase. Then, cells were stained with PI and then immediately analyzed by a 488 nm single laser emitting FACSCalibur (BD biosciences) within 15 minutes.

2.5. REAL-TIME PCR

Forward and reverse primers for isocitrate dehydrogenase (NADP(+)) 1 (IDH1), poly(ADP-ribose) polymerase 1 (PARP1), poly(ADP-ribose) polymerase 2 (PARP2), Caspase-3, Caspase 7, Antigen KI-67 (Ki-67 or MKI67), Proliferating cell nuclear antigen (PCNA), Tumor protein 53 (TP53), Breast Cancer Gene 1 (BRCA1), Breast Cancer Gene 2 (BRCA2), Ataxia telangictasia mutated (ATM), Ataxia telangictasia and Rad3 related (ATR), cyclin dependent kinase inhibitor 1A (CDKN1A), Cyclin Dependent Kinase 1 (CDK1), Cyclin

Dependent Kinase 2 (CDK2), Cyclin Dependent Kinase 4 (CDK4), Cyclin Dependent Kinase 6 (CDK6), Beclin, Autophagy Related 5 (ATG5), Microtubule-associated Protein 1A/1B Light Chain 3A (LC3A), Inducible heat shock protein A1A (HSP1A). The expression levels were normalized with respect to subunit of mitochondrial ATP synthase (ATP-5) gene and all genes designed by using Primer-BLAST software (National Center for Biotechnology, Bethesda, MD, USA) (Table 2.5). The RNA isolation kit (#740955.250, Macherey-NAGEL, Düren, Germany) was used to extract total RNA in accordance with the instructions in the user's manual. The QuantiTect Reverse Transcription Kit (#205313, QIAGEN, Hilden, Germany) was then used to transform extracted total mRNAs into cDNAs. The iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA) was used to perform RT-PCR using SYBR Green (#4309155, Thermo Fisher, Waltham, ABD).

Table 2.5. Using gene pairs with forward and reverse primers.

Gene	**Side**	**Sequence**
Survivin	F	5’TCATGCTGAGGCTCCAGAGTTC3’
	R	5’ACAGTCTTGCCAACTCCAGCAC3’
CDKN1A	F	5’ACATGTGCACGGAAGGACTTTG3’
	R	5’CGTTTGGAGTGGTAGAAATCTGGAG3’
ATG5	F	5’AAAGATGTGCTTCGAGATGTGT3’
	R	5’CACTTTGTCAGTTACCAACGTC3’
	F	5’GGTGTCTCTCGCAGATTCATC3’

BECLIN		
	R	5’TCAGTCTTCGGCTGAGGTTCT3’
LC3B	F	5’AAGGCGCTTACAGCTCAATG3’
	R	5’CTGGGAGGCATAGACCATGT3’
LC3A	F	5’AACATGAGCGAGTTGGTCAAG3’
	R	5’GCTCGTAGATGTCCGCGAT3’
HSP1A	F	5’GCGATGGTCAATGGTCAT3’
	R	5’GACCGTTAGTTCAGGCATG3’
Caspase 8	F	5’GCCACCCGGCTTCAGAATGGC 3’
	R	5’TATGGGCCATCTGCTGTTGGCAGT 3’
Caspase 7	F	5’ TCAGTGGATGCTAAGCCAGACC 3’
	R	5’ CGAACGCCCATACCTGTCAC 3’
PARP1	F	5’CCAAGCCAGTTCAGGACCTCAT3’
	R	5’GGATCTGCCTTTTGCTCAGCTTC3’
	F	5’CAGGAAGGTCATGGGCCAG3’

PARP2		
	R	5’TGTCCCATTTTCCCAACTCGG3’
BCL2	F	5’ AACGGAGGCTGGGATGCCTTTGTG 3’
	R	5’ ACCAGGGCCAAACTGAGCAGAGT 3’
BAX	F	5’TGCAGAGGATGATTGCCGCCG3’
	R	5’ACCCAACCACCCTGGTGTTGG3’
Ki-67	F	5’ GAAAGAGTGGCAACCTGCCTTC 3’
	R	5’ GCACCAAGTTTTACTACATCTGCC 3’
PCNA	F	5’ CAAGTAATGTCGATAAAGAGGAGG 3’
	R	5’ GTGTCACCGTTGAAGAGAGTGG 3’
BRCA1	F	5’GAACCAGGAGTGGAAAGGTCA3’
	R	5’GCTGTTGCTCCTCCACATCA3’
BRCA2	F	5’AGACTGTACTTCAGGGCCGTACA3’
	R	5’GGCTGAGACAGGTGTGGAAACA3’

TP53	F	5’ GCCCAACAACACCAGCTCCT 3’
	R	5’ CCTGGGCATCCTTGAGTTCC 3’
ATM	F	5’ TGTTCCAGGACACGAAGGGAGA 3’
	R	5’ CAGGGTTCTCAGCACTATGGGA 3’
ATR	F	5’ GGAGATTTCCTGAGCATGTTCGG 3’
	R	5’ GGCTTCTTTACTCCAGACCAATC 3’
CDK1	F	5’ CACTTGGCTTCAAAGCTGGCTC 3’
	R	5’ ATGGGTATGGTAGATCCCGGC 3’
CDK2	F	5’ CTGGACACGCTGCTGGATG 3’
	R	5’ ATGCCAGTGAGAGCAGAGGC 3’
CDK4	F	5’ GTCTATGGTCGGGCCCTCTG 3’
	R	5’ CAGATCAAGGGAGACCCTCACG 3’
CDK6	F	5’ GTCTGATTACCTGCTCCGCGA 3’
	R	5’ TCCAGAATCATTGCACCTGAGGG 3’

IDH-1	F	5’ GTCTGATTACCTGCTCCGCGA 3’
	R	5’ TCCAGAATCATTGCACCTGAGGG 3’
ATP5B	F	5’TCATGCTGAGGCTCCAGAGTTC 3’
	R	5’ACAGTCTTGCCAACTCCAGCAC 3’

2.6. ETHYNYL-2′-DEOXYURIDINE ASSAY

IDH-MUT-U87 and U373MG cells were seeded in 4 wells of a Millicell® EZ Slide (4-well), and following one day, the cells were treated with NaB, Vitmain-C, and their combinations alone and in combination. Additionally, cells were given treatment with a culture media including 20 M EdU reagent after 72 hours. The cells were then fixed by paraformaldehyde after being cultured for 2 hours. DAPI was used to stain nuclei. Time-lapse fluorescence microscopy was used to measure and examine the percentage of EdU-positive cells. The manufacturer's recommendations were followed while using the EdU Staining Proliferation Kit (iFluor 647) (Abcam, Cambridge, MA, UK; ab222421).

2.7. SCRATCH ASSAY

IDH-MUT-U87, and U373MG cell lines were seeded in the six-well plate (#CLS3512, Corning Plasticware, Corning, NY) and their densities are $2x10^5$ cells/well. They were incubated for 24 hours in the incubator. After 24 hours, the cells were scratched. Then, the media was aspirated and the cell was washed with 1 ml PBS. fresh media containing alone NaB, Vitamin-C and their combination was added and each good photograph was taken first hours and 72 hours.

2.8. ICC (IMMUNOCYTOCHEMISTRY)

After 72 hours, the IDH-MUT-U87 and U373MG cells were fixed with 2% paraformaldehyde for 30 minutes at 4°C using a Millicell® EZ Slide, 4-well. To permeabilize cells at room temperature, 0.1% Triton-X100 was then applied to each well for 10 minutes. Each well received 10% FBS administered to inhibit cells. After that, cells were treated overnight with the primary antibodies 1:1000 for ATG-5 (ab108327), LC3B/A (ab32064) and PARP1 (D64E10). A secondary antibody was then applied to the cells and incubated for 2 hours. Each well received a 1:1000 solution of the dye 4′,6-diamidino-2-phenylindole (DAPI) for 20 minutes at 4°C. Using confocal microscopy, the proportion of labeled cells was measured and examined.

2.9. DCFA/H2DCFDA CELLULAR ROS ASSAY

In T25 flasks, 75 x 104 and 10 x 105 IDH-MUT-U87 and U373MG cells, respectively, were planted. The next day, cells were collected, stained with 20 M DCFDA, and incubated at 37°C for 30 minutes. Cells were centrifuged and PBS washed after that. NaB, vitamin C, and their combination were applied to the cells. Flow cytometer FACSCalibur (BD Biosciences) was used to assess ROS levels in the end.

2.10. STATISTICALLY ANALYSIS

All data are demonstrated as the means ± standard errors. Graphs were created using the GraphPad Prism 5 program, and an unpaired t-test was used for the statistical analysis of the data. The threshold for statistical significance was set at $p < 0.05$.

3. RESULT

3.1. THE EFFECT OF NaB, OLAPARIB, TEMOZOLOMIDE TOGETHER WITH VITAMIN-C ON CELL VIABLITY

Toxic effects of Vitamin-C, NaB, Olaparib and Temozolomide was detected for IDH-mut-U87, U373MG cancer cell lines and NHA normal human astrocytes cell lines either alone or their combination with Vitamin-C at selected range of concentration for 24,48,72 hours. IC50 values at 24, 48, and 72 hours for all treatment groups were determined in IDH-mut-U87, U373MG and NHA cell lines by using an MTS assay (Table 3.1).

The results showed that IC50 values of NaB treatments was determined 1250 μg/ml both in IDH-mut-U87 and U373MG cancer lines while in normal human astrocytes NHA cell lines is 5000 μg/ml. Thus, NaB treatments reduce cell viability in cancer cell lines, but in NHA (astrocytes cell lines), toxic effect was not detected (Figure 3.1.1b (U373MG), Figure 3.1.3b (IDH-MUT-U87) and Figure 3.1.5b (NHA)). In addition, Vitamin-C non-toxic doses was applied in combination treatments for showing synergetic effect of Vitamin-C and other compounds. Vitamin-C non-toxic doses was determined 350 μg/ml, 500 μg/ml for IDH-mut-U87 and U373MG cell lines respectively, although, the highest high doses (500 μg/ml) was used non-toxic dose in cancer cell lines was applied to NHA cell lines. As a result, in this experiment, combination with NaB and Vitamin-C, cell viability was reduced by five times for IDH-mut-U87 cancer cell lines and the combination was not significantly effective for U373MG cancer cell lines. In addition, effective doses of both cancer cell lines was showed no cell toxic effect when used in NHA cell lines (Figure 3.1.6a).

According to the findings, IC50 values of TMZ treatments was determined 1mM for both cell lines IDH-mut-U87 and U373MG. Remarkably, TMZ and Vitamin-C combination was decreased approximately by 2 times in U373MG cell lines even though, same combination was not crucially effective IDH-mut-U87 cancer cell lines. Also, combination of TMZ and Vitamin-C was found to be toxic in astrocytes NHA cell lines compared to both cancer cell lines.

Alone Olaparib IC50 values was determined same doses (0.5mM) for IDH-mut-U87 cancer cell lines and astrocytes NHA cell lines. Furthermore, combination of Olaparib and

Vitamin-C was decreased cell viability by 2 times while these combinations did not display cytotoxicity in NHA cell lines. Also, alone and their combination of TMZ was not effective or synergetic effects in U373MG cell lines.

Table 3.1. IC50 values of IDH-mut-U87, U373MG and NHA cell lines for each treatment at 72hours

Treatment	IC50 values of IDH-mut U87	IC50 values of U373	IC50 values of NHA
Vitamin-C	450 µg/ml	1000-500 µg/ml	2000 µg/ml
NaB	1250 µg/ml	1250 µg/ml	5000 µg/ml
NaB+VitC	250 µg/ml	1000 µg/ml	2500 µg/ml
Olaparib	0.5 mM	1 mM	0.5 mM
Olaparib+VitC	0.25 mM	0.5-0.25 mM	1 mM
TMZ	1 mM	1 mM	0.5 mM
TMZ+VitC	~ 0.8 mM	0.5-0.25 mM	0.25 mM

Table 3.2. Vitamin C application in non-toxic doses for each cell lines

Cell Lines	Vitamin-C Application at non-toxic doses
IDH-mut-U87	350 µg/ml
U373MG	500 µg/ml
NHA	500 µg/ml

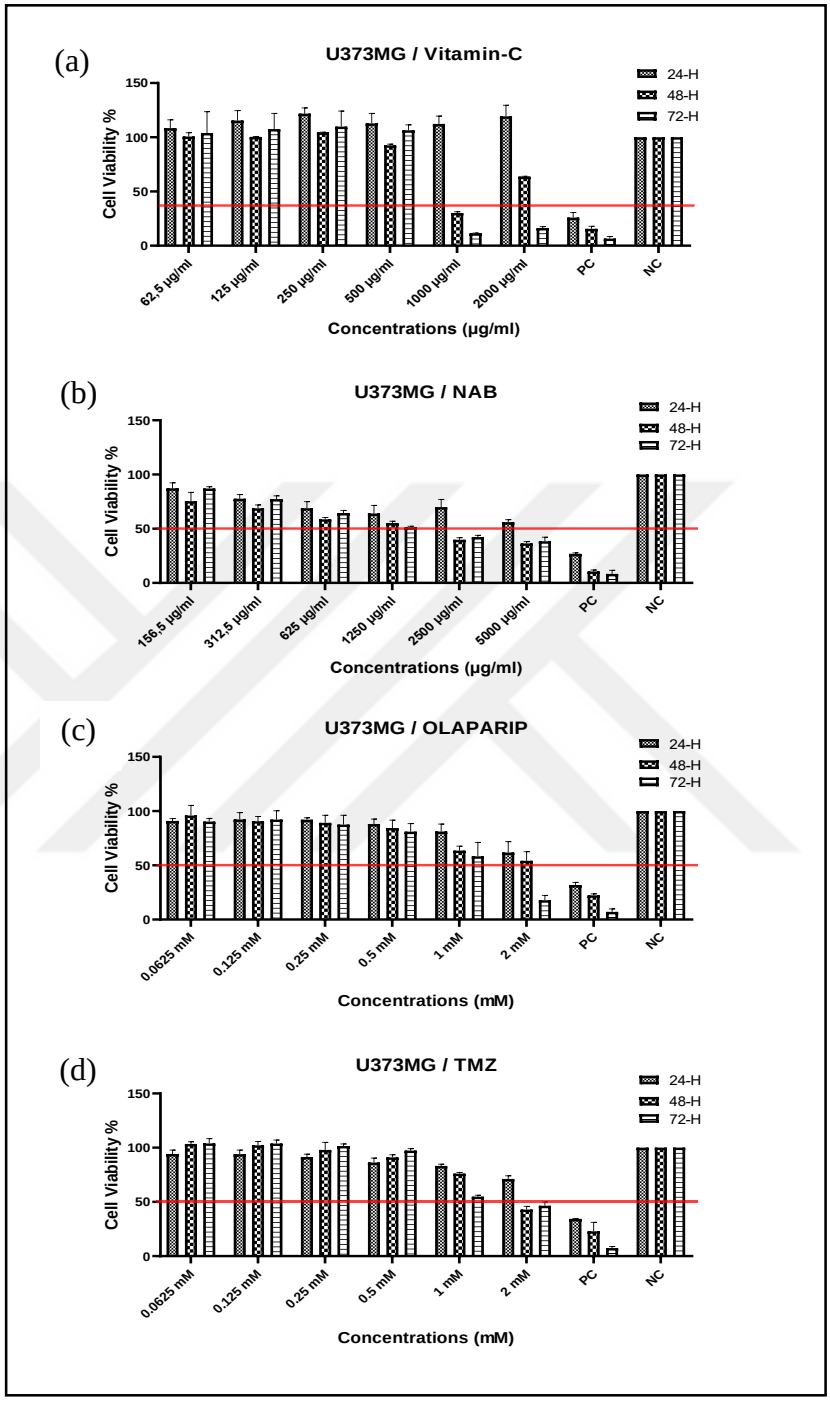

Figure 3.1.1. The cell viability rates (percent) of U373MG cancer cell lines at 24, 48, 72 hours with (a) Vitamin-C (b) NaB (c) Olaparib (d) Temozolomide (TMZ).

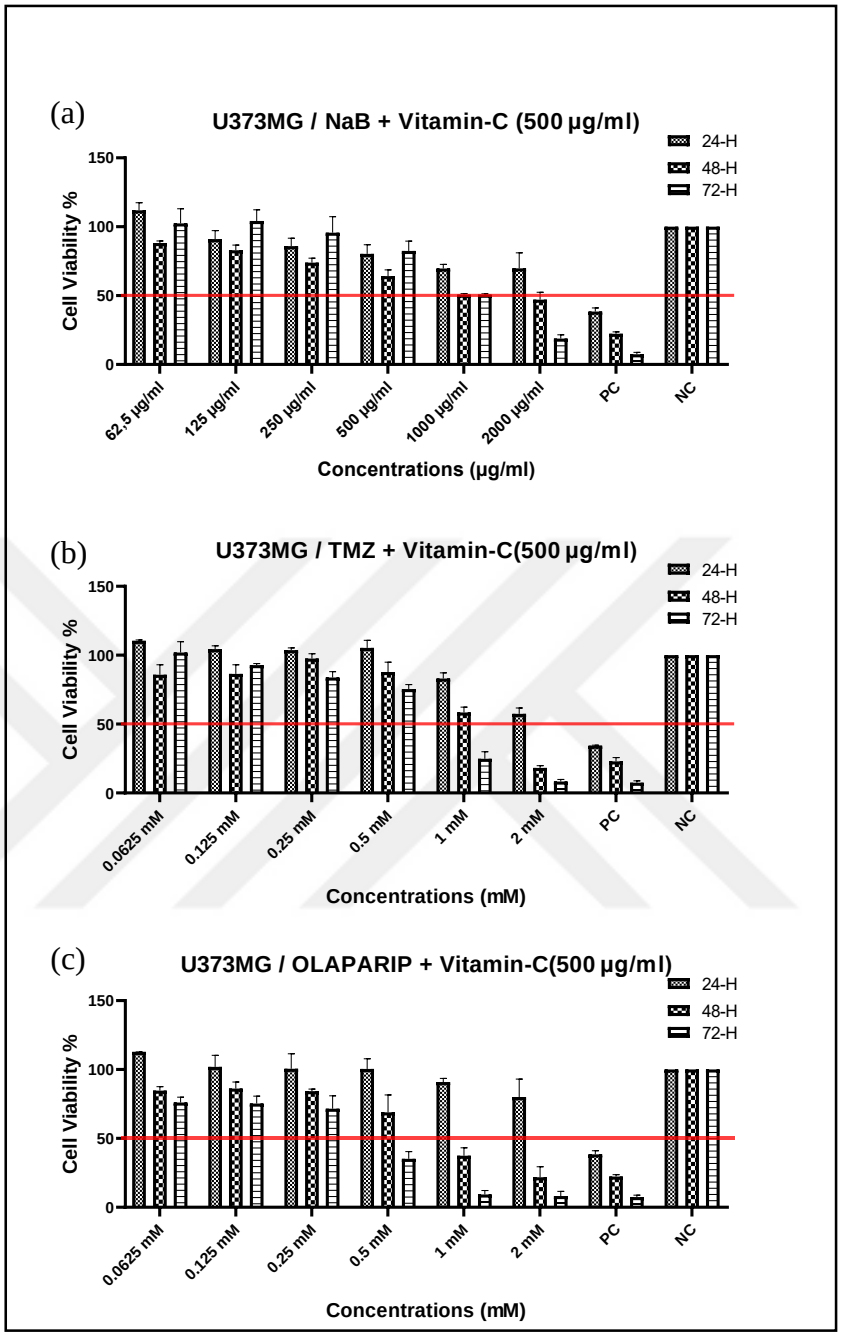

Figure 3.1.2. The cell viability rates (percent) of U373MG cancer cell lines at 24, 48, 72 hours with (a) combination of NaB and Vitamin-C (b) combination of TMZ and Vitamin-C (c) combination of olaparib and Vitamin-C.

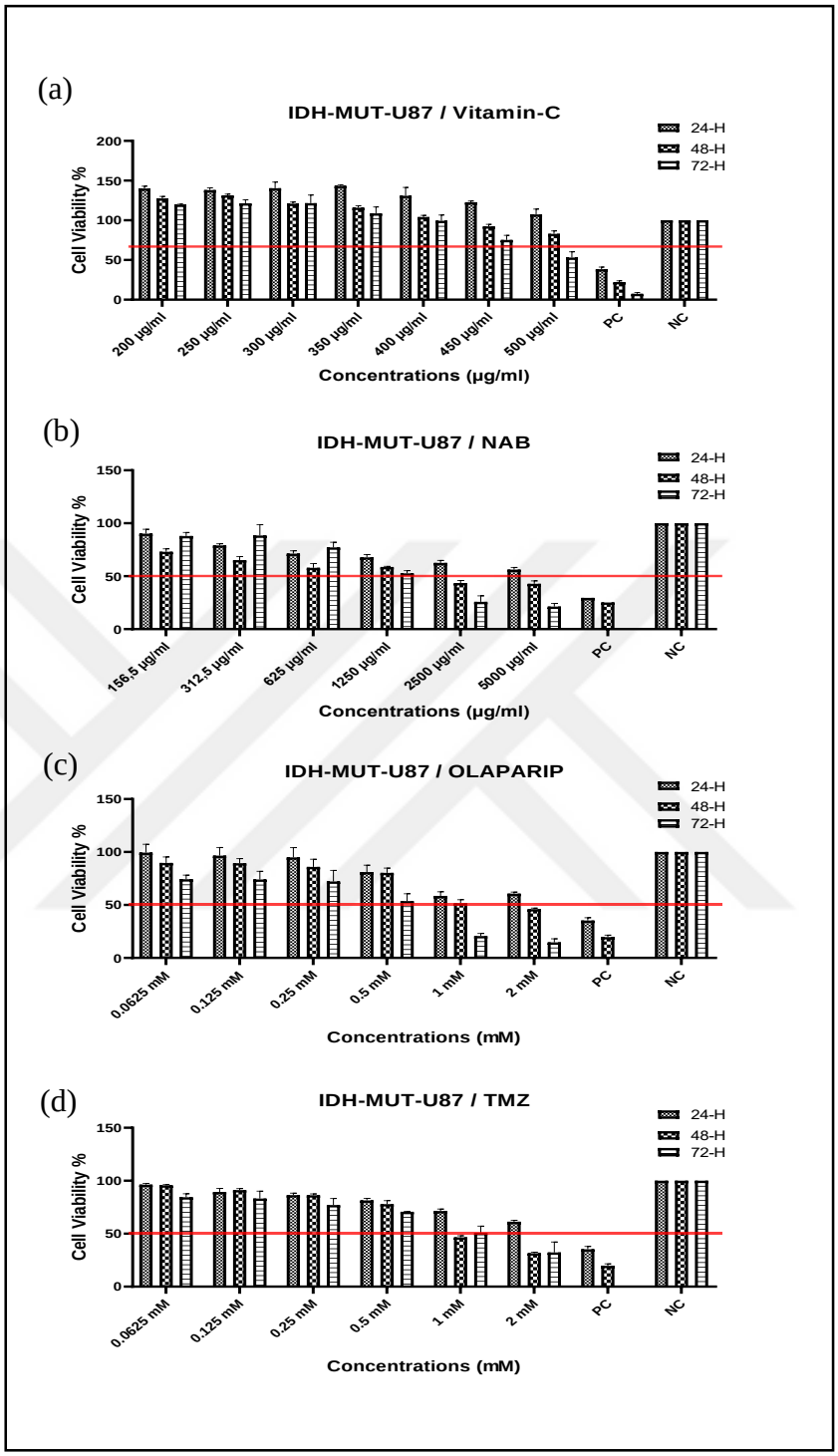

Figure 3.1.3. The cell viability rates (percent) of IDH-MUT-U87 cancer cell lines at 24, 48, 72 hours with (a) Vitamin-C (b) NaB (c) Olaparib (d) Temozolomide (TMZ).

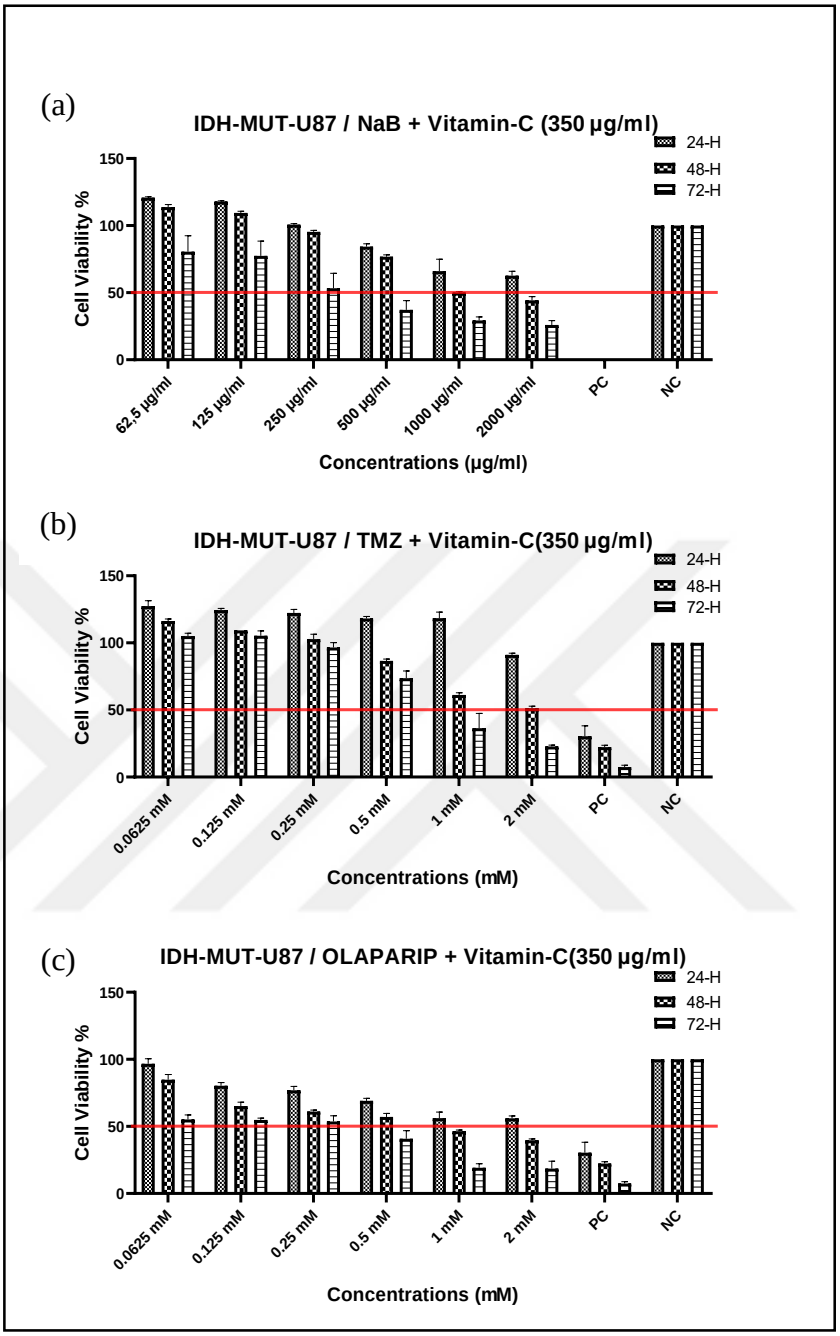

Figure 3.1.4. The cell viability rates (percent) of IDH-MUT-U87 cancer cell lines at 24, 48, 72 hours with (a) combination of NaB and Vitamin-C (b) combination of TMZ and Vitamin-C (c) combination of olaparib and Vitamin-C.

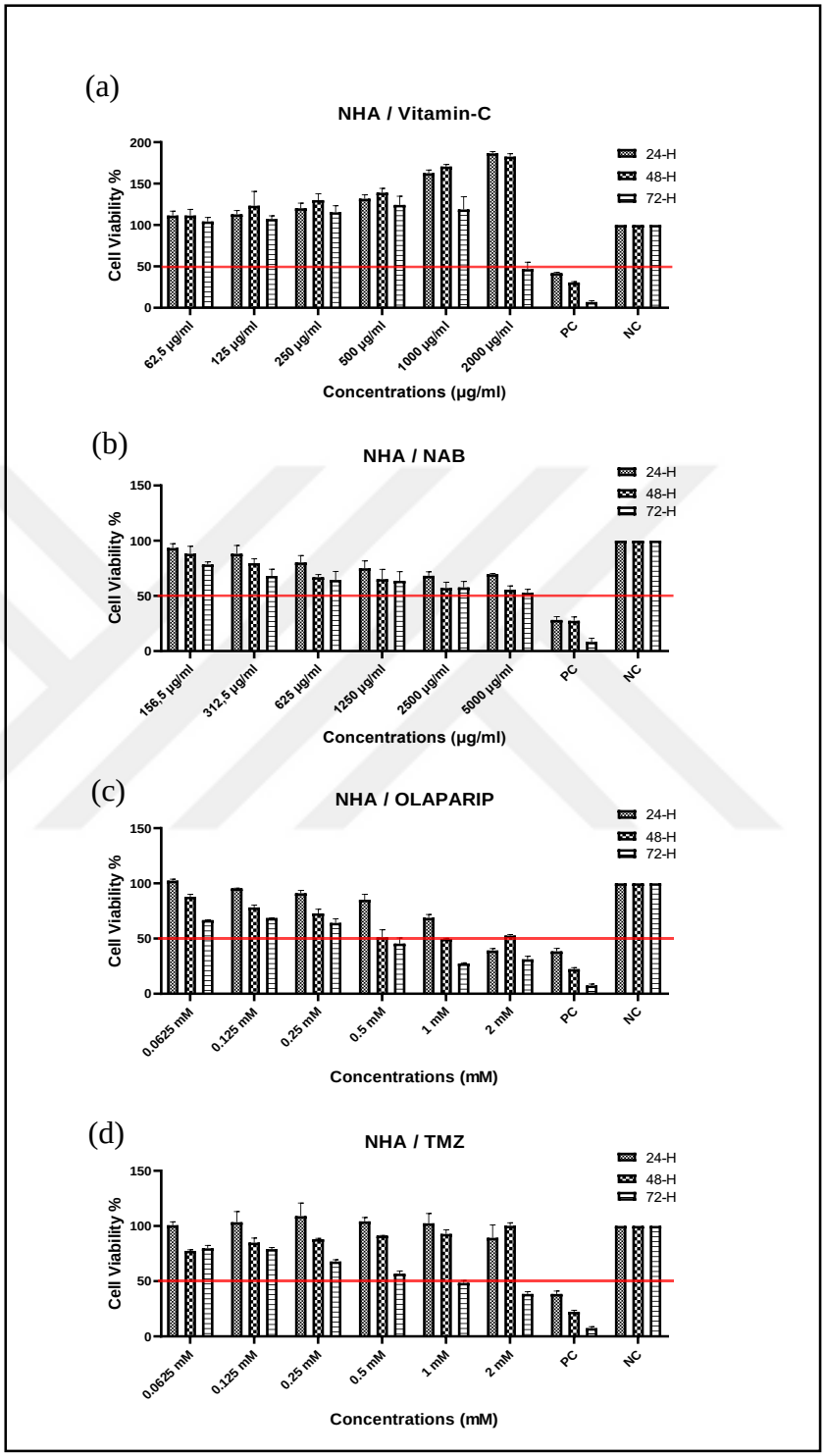

Figure 3.1.5. The cell viability rates (percent) of NHA astrocytes cell lines at 24, 48, 72 hours with (a) Vitamin-C (b) NaB (c) Olaparib (d) Temozolomide (TMZ).

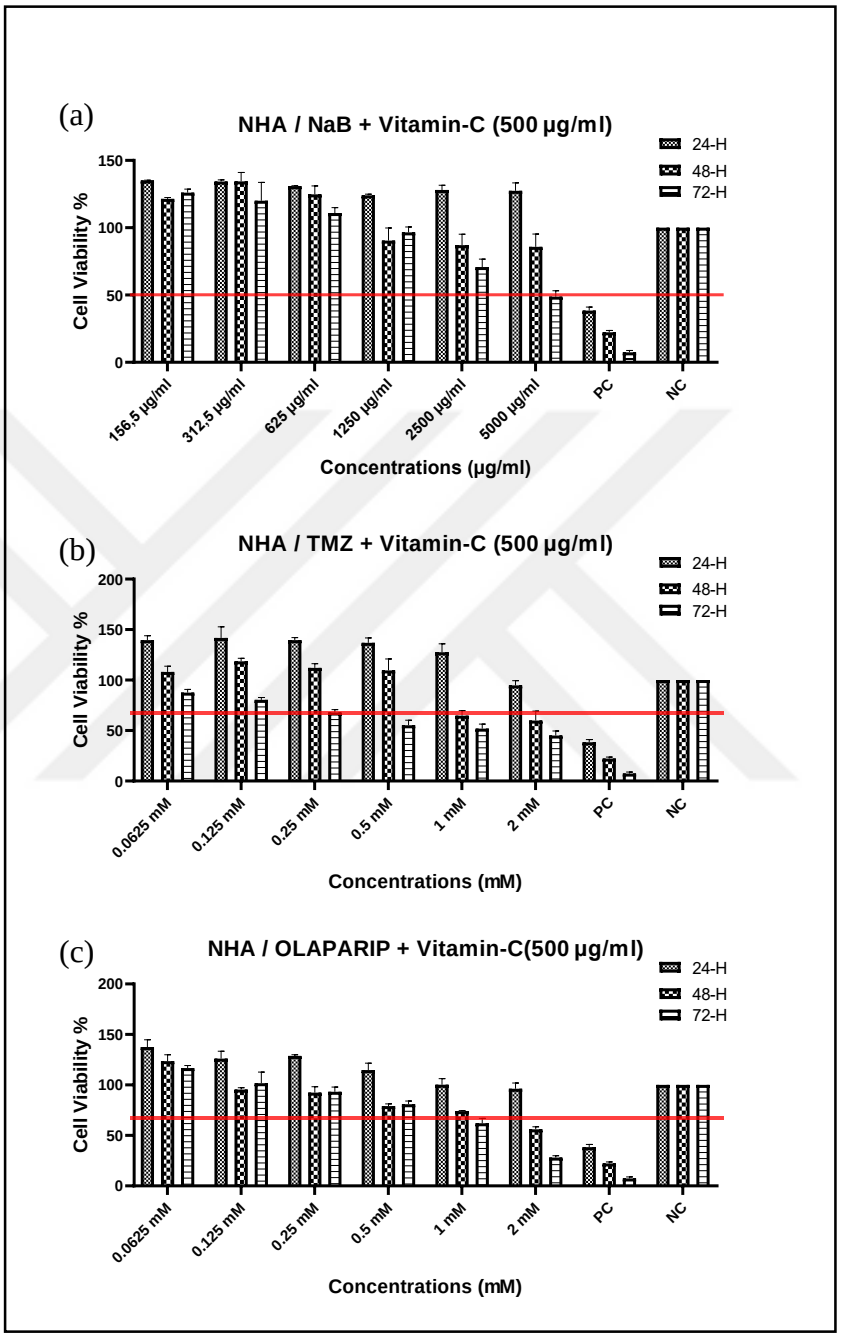

Figure 3.1.6. The cell viability rates (percent) ofNHA cancer cell lines at 24, 48, 72 hours with (a) combination of NaB and Vitamin-C (b) combination of TMZ and Vitamin-C (c) combination of olaparib and Vitamin-C.

3.2. THE EFFECT OF NaB AND ITS COMBINATON WITH VITAMIN-C ON APOPTOSIS

The effect of NaB, Vitamin-C and their combination on cell death pathway apoptosis was determined in IDH-mut-U87 and U373MG cancer cells by using the Annexin-V assay. The assay was carry out at IC50 values of NaB and their combination of NaB and Vitamin-C while alone Vitamin-C treatments was performed at non-toxic doses for IDH-mut-U87 and U373MG cell lines. In the cytograms, bottom left quadrant shows live cells and upper left quadrant shows necrotic cells that are PI positive (Figure 3.2.1 and Figure 3.2.2).

Overall, alone NaB treatment was not induced the apoptosis of both IDH-mut-U87 and U373MG cancer cell lines compared to the negative control. Also, in the U373MG cell, both NaB alone and in combination with vitamin C had any effect on apoptosis and necrosis (Figure 3.2.1). However, combination of NaB and Vitamin-C application was resulted in extensive effect of late apoptosis in IDH-mut-U87 cell lines (NaB+Vitamin-C: 6.08% for early apoptosis marker, 36.57% for late apoptosis marker and 1.42% for necrosis marker) (Figure 3.2.2).

In order to understand the exact mechanism underlying the apoptosis induced by the NaB, Vitamin-C and their combination used in this study, the expression levels of important genes involved in programmed cell death were examined. In mammalian cells, CASP7 have been identified as important apoptosis executors and are essential for this phase of the process [123,124]. The cysteine protease CASP8 starts the apoptotic cascade and provides apoptotic signals after death receptors by cleaving downstream substrates such effector CASPs. Also, Expression levels of Casp7 and Casp8 gene were increased in response all treatment (≈ 2.65 fold for NaB, ≈ 1.82 fold for Vitamin-C and ≈ 2.11 fold for combination of NaB+CVIT) in IDH-MUT-U87 although casp7 genes was decreased in U373MG cell lines compared to negative control. However, Casp8 genes was elevated in response to NaB (≈ 1.08 fold) and combination of NaB and Vitamin-C (≈ 1.40 fold) but casp8 genes decrease in alone Vitamin-C (≈ 0.74 fold) treatments. Tp-53 is a tumor suppressor gene that, can induce apoptosis by activation of BH3-only proteins, Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) and p53 upregulated modulator of apoptosis (PUMA) genes [125]. There were significant changes in the TP53 expression when IDH-MUT-U87 cells were treated with NaB (≈ 4.04 fold), Vitamin-C (≈ 1.53 fold) and their combination (≈ 1.91 fold) (Figure

3.2.3b) even though Tp53 was decreased for all treatment in U373MG (Figure 3.2.3a) cell lines. Another apoptosis gene of BAX and BCL2 was no significant changes when IDH-MUT-U87 cells were treated all treatments.

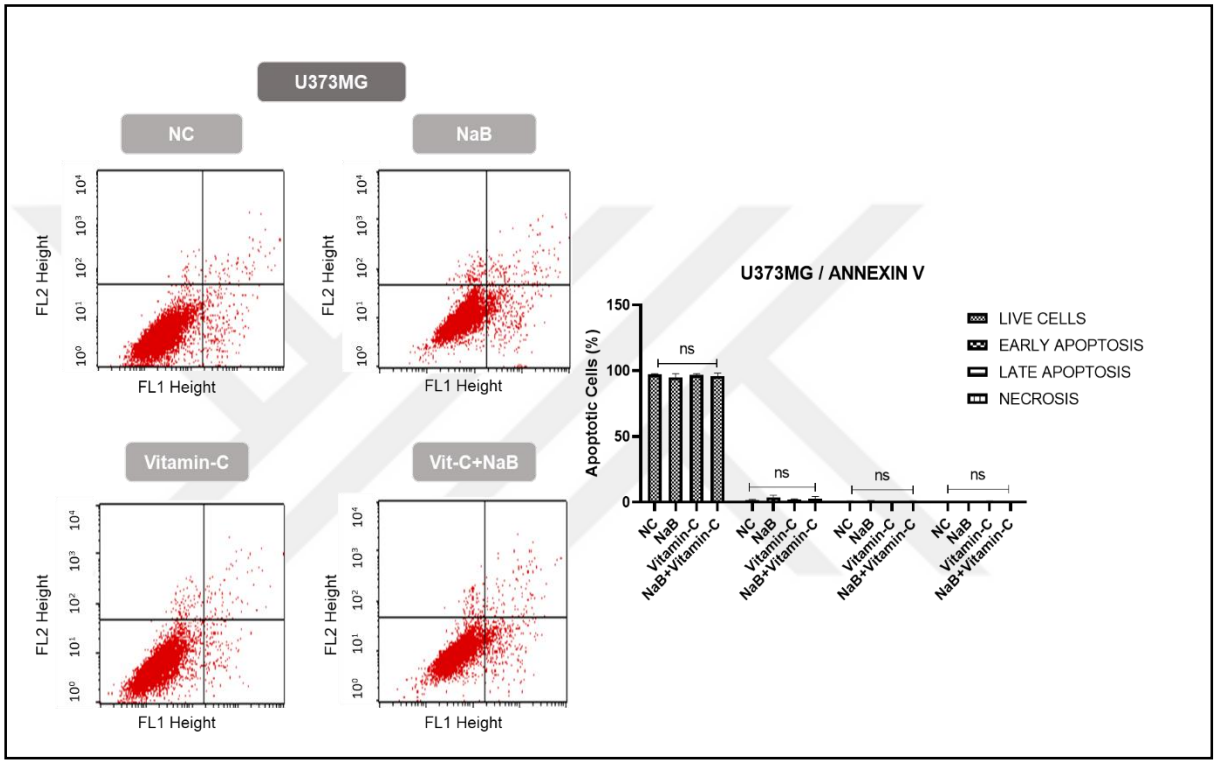

Figure 3.2.1. Result of Annexin-V staining for U373MG cell lines at 72 h**.**

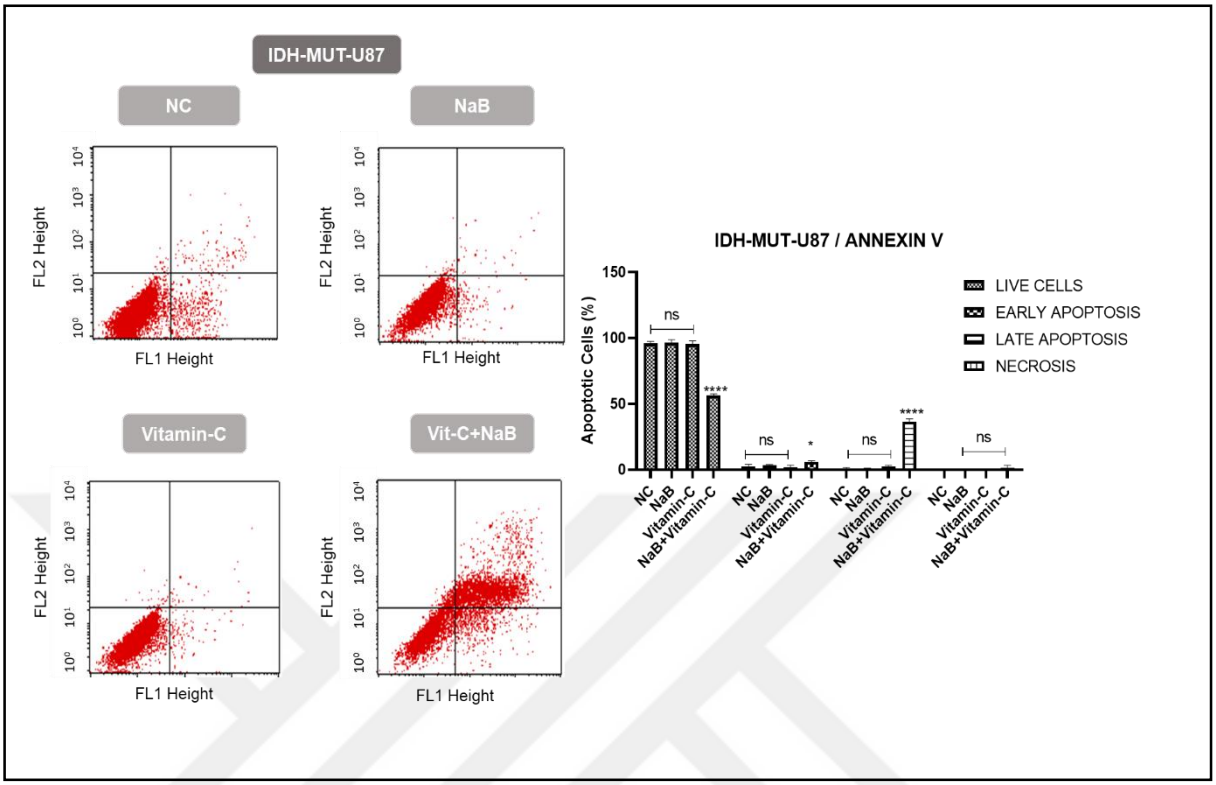

Figure 3.2.2. Result of Annexin-V staining for IDH-MUT-U87 cell lines at 72 h.

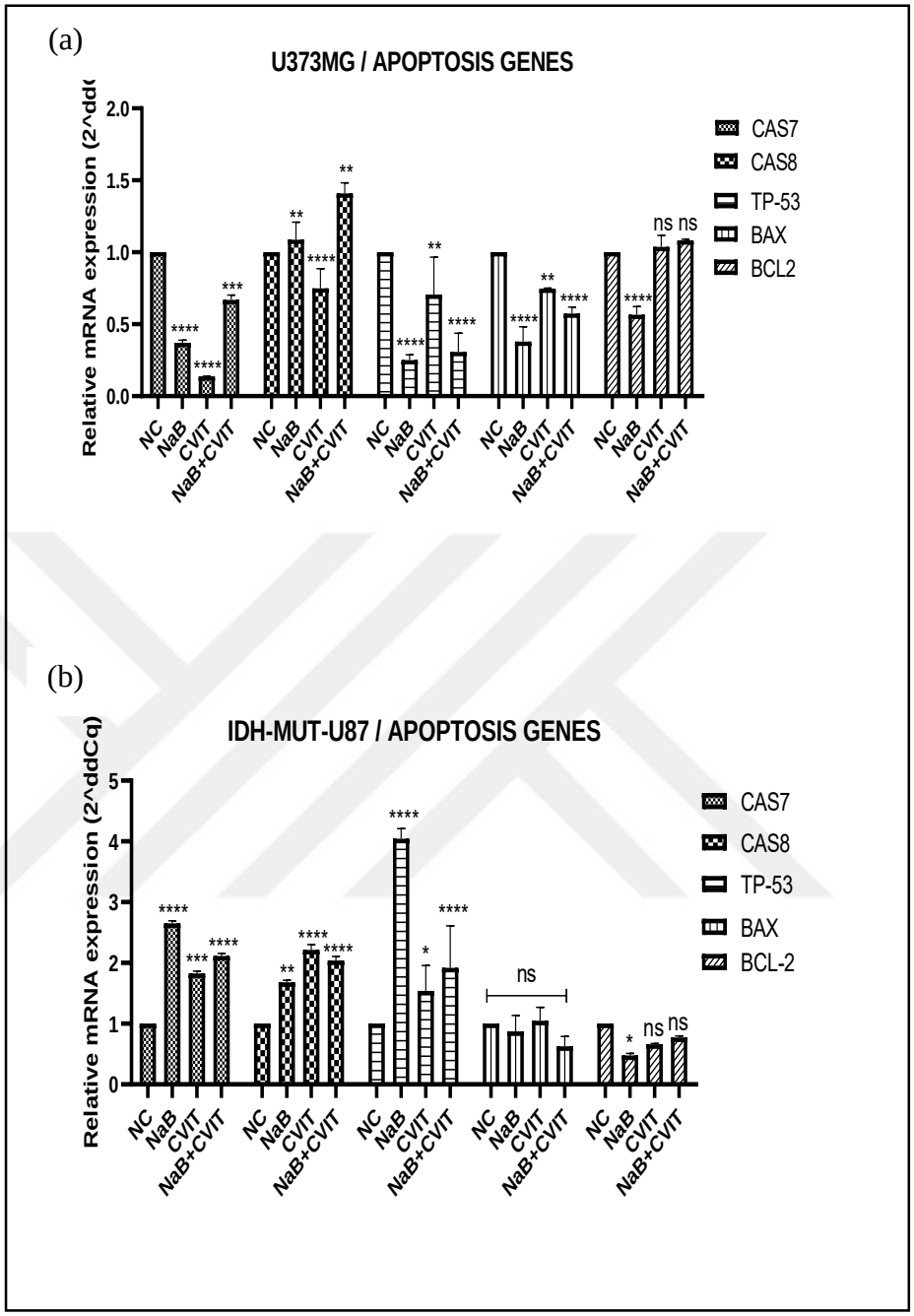

Figure 3.2.3. Apoptotic genes of mRNA expression in (a) U373MG and (b) IDH-MUT-U87cells after 72 h.

3.3.THE EFFECT OF NaB AND ITS COMBINATON WITH VITAMIN-C ON CELL CYCLE AND CELL PROLIFERATION

To verify the NaB and its combination with Vitamin-C cell cycle effect on the IDH-mut-U87 and U373MG cell lines were exposed for 3 days. Also, both cancer cell lines were obtained using same treatment procedure used in apoptosis assay for cell cycle profiles. In the Vitamin-C combination of IDH-mut-U87 cell lines was significantly increased in the cell numbers in G2 phase (%14.13) compared to negative control (Figure 3.3.2). On the other hand, in U373MG cell lines both alone Vitamin-C and their combination with NaB were significantly increased in the cell numbers in S phase (23.26% for Vitamin-C treatments, 32.52% for Vitamin-C+NaB treatments) compared to negative control.

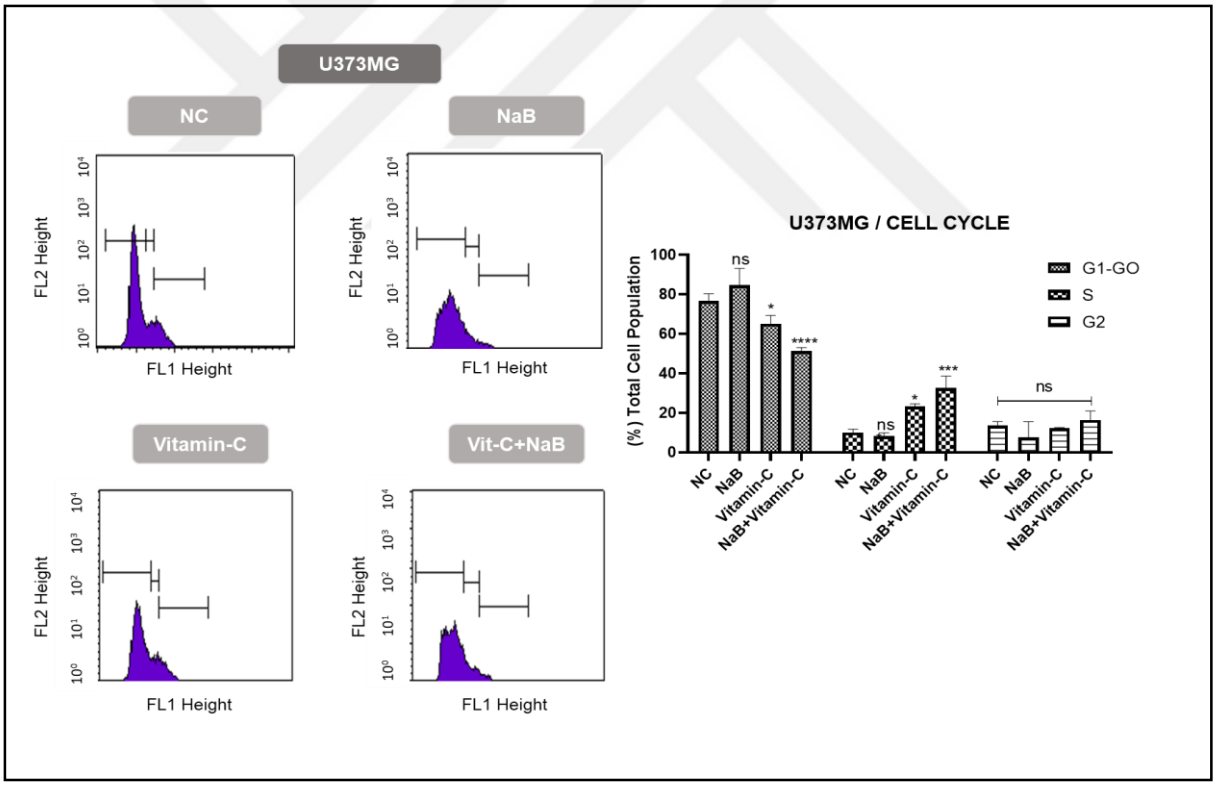

Figure 3.3.1. Cell cycle analysis using flow cytometry for U373MG cells.

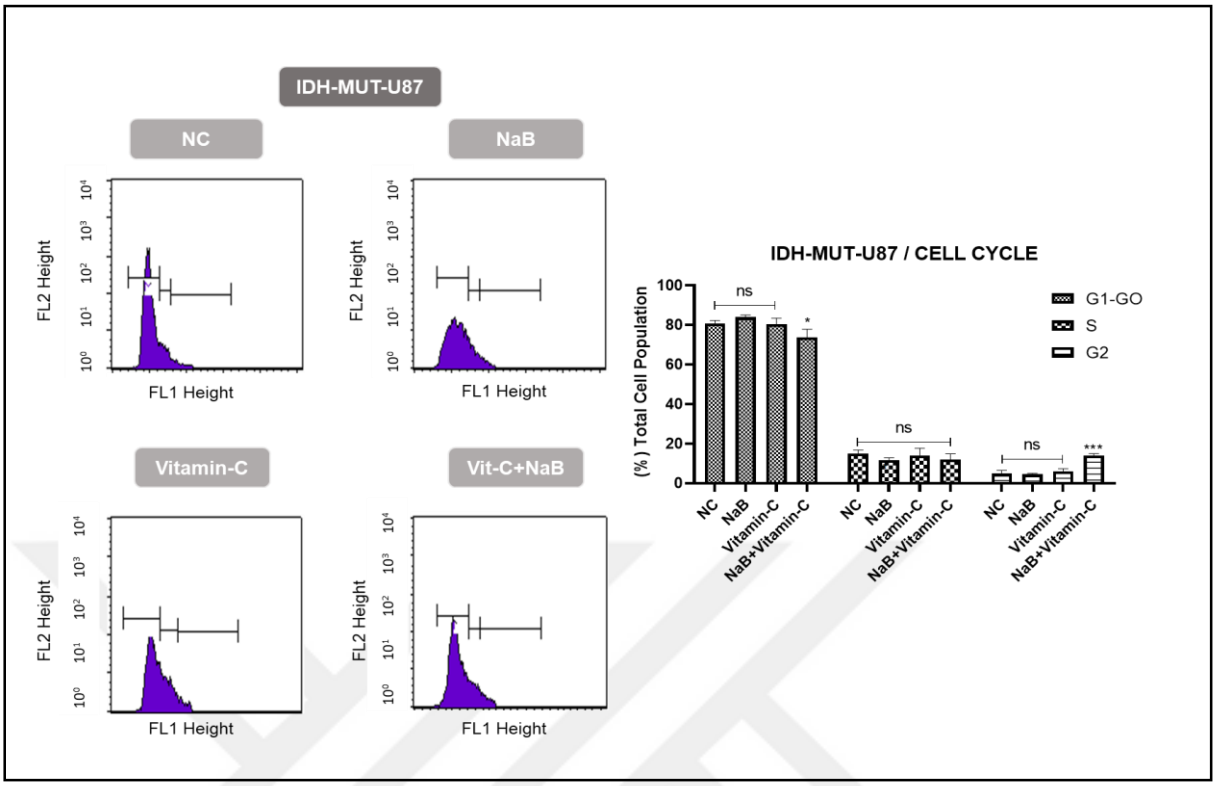

Figure 3.3.2. Cell cycle analysis using flow cytometry for IDH-MUT-U87 cells

Nevertheless, Cyclin Dependent Kinase 1 (CDK1), Cyclin Dependent Kinase 2 (CDK2) Cyclin Dependent Kinase 4 (CDK4), Cyclin Dependent Kinase 6 (CDK6) genes are central regulator of cell cycle and their expression levels were determined both in IDH-MUT-U87 and U373MG cell lines under NaB, Vitamin-C and their combination treatment (Figure 3.3.3). In both of IDH-mut-U87 and U373MG cells, while CDK4, and CDK6 expression levels were not changed with any treatments. However, CDK2 genes expression levels were upregulated upon combination of Vitamin-C and NaB (≈ 5.32 fold and ≈ 2.63 fold) in IDH-MUT-U87 and U373MG cell lines respectively (Figure 3.3.3a and Figure 3.3.b). Also, CDK1 levels was decreased ≈ 0.23 fold and ≈ 0.12 fold when combination treatment in U373MG and IDH-MUT-U87 cancer cell lines respectively (Figure 3.3.3a and Figure 3.3.b).

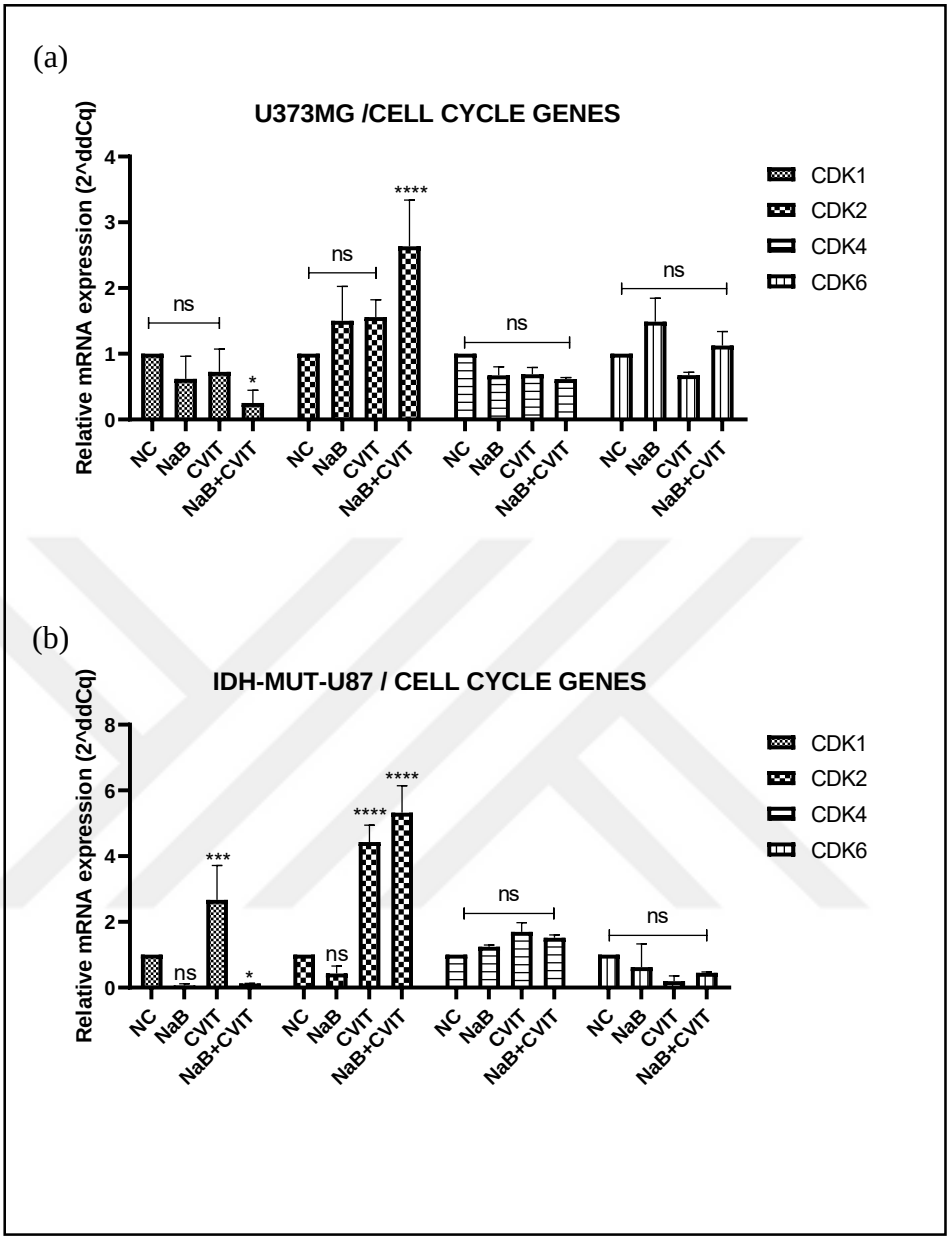

Figure 3.3.3. Cell cycle genes of mRNA expression in (a) U373MG and (b) IDH-MUT-U87 cells after 72 h.

Changes in cell proliferation and mechanisms between IDH-mut-U87 and U373MG cell lines were analyzed by using real-time PCR. Real- time PCR experiment were significantly downregulated that (Marker of Proliferation) Ki-67 (≈0.5 fold for both NaB and NaB+CVIT), (Proliferating Cell Nuclear Antigen) PCNA (≈0.28 fold for NaB and ≈0.18 fold for NaB+CVIT), and (Baculoviral IAP Repeat Containing 5) Survivin (≈0.16 fold for NaB and ≈0.18 fold for NaB+CVIT) with alone NaB and combination of NaB and Vitamin-C in U373MG cell lines. Also, no sigbificant changes was examined for these genes expression levels in IDH-MUT-U87 cells but, CDKN1A (≈0.16 fold) expression level was increased with combination of NaB and Vitamin-C.

The effect of NaB and Vitamin-C and their combination on the cell proliferation of IDH-MUT-U87 and U373MG cells was investigated by DNA staining-based assay (Figure 3.3.5 and Figure 3.3.6). Moreover, cellular proliferation was clearly suppressed DNA proliferation with combination treatment compared to negative control in both cell lines. However, in IDH-MUT-U87 cell, DNA proliferation was significantly increased upon treatment of NaB.

The effect of NaB, Vitamin-C and their combination on cell migration ability was observed in scratch assay upon GBM cancer cells. As a result of the scratch assay, cell migration capacities was significantly decreased with combination treatments in IDH-MUT-U87 cells (Figure 3.3.6). On the other hand, although cell proliferation was not changed compared to negative control, there has been a noticeable difference in cell shapes such as rounder and cell size is smaller in U373MG.

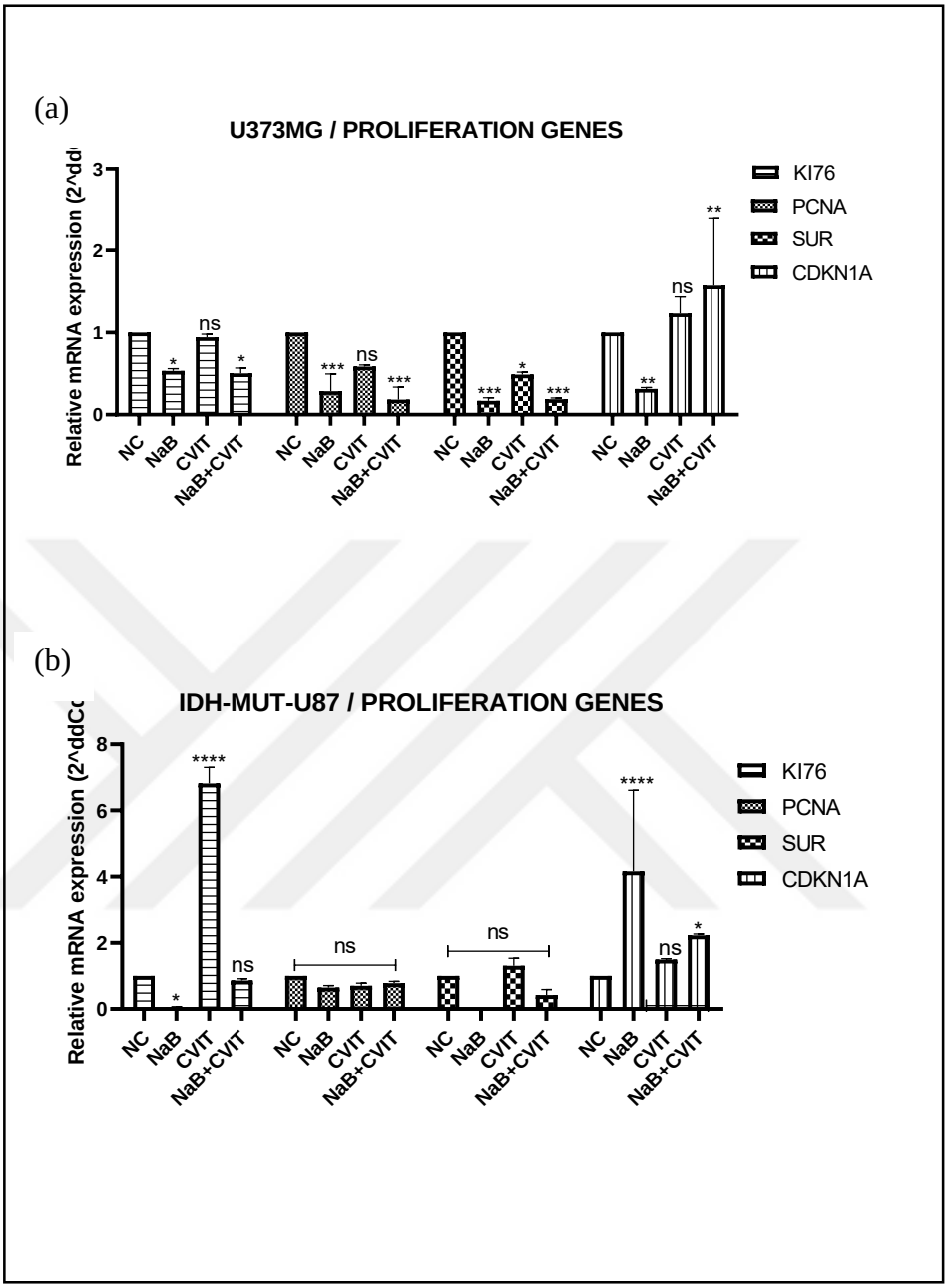

Figure 3.3.4. Cell proliferation genes mRNA expression in (a) U373MG and (b) IDH-MUT-U87 cells after 72 h.

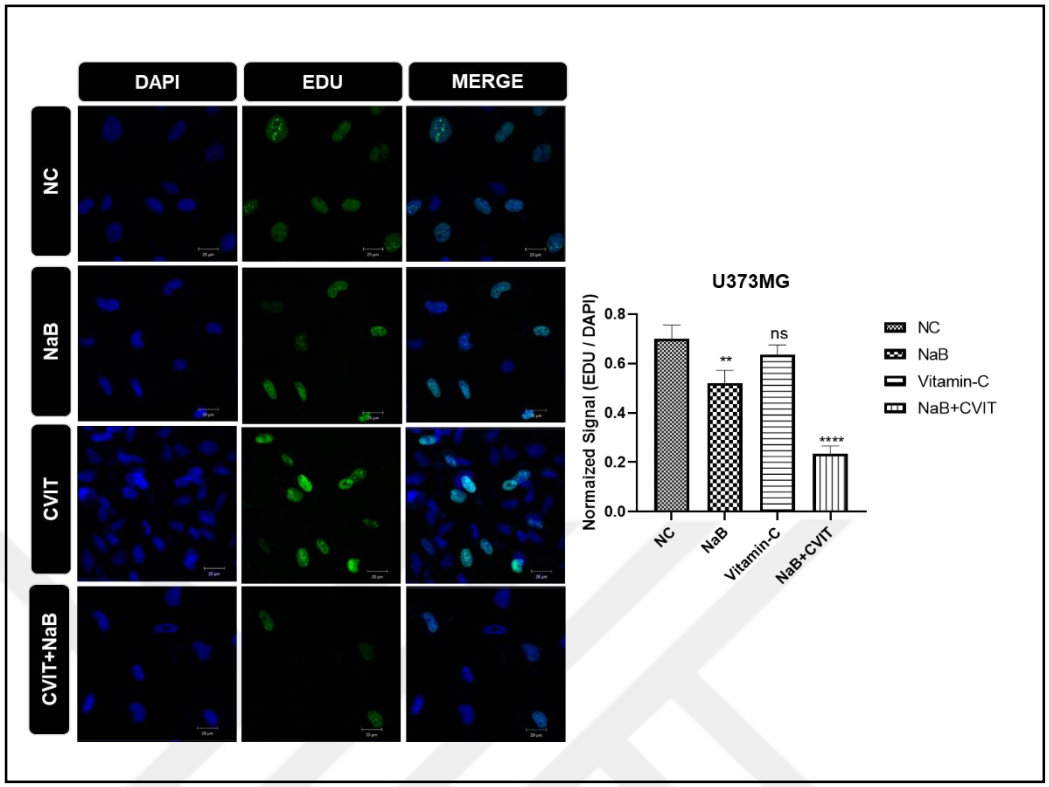

Figure 3.3.5. EDU assay result for U373MG cell line. EDU green; dividing cell, blue; DAPI staining.

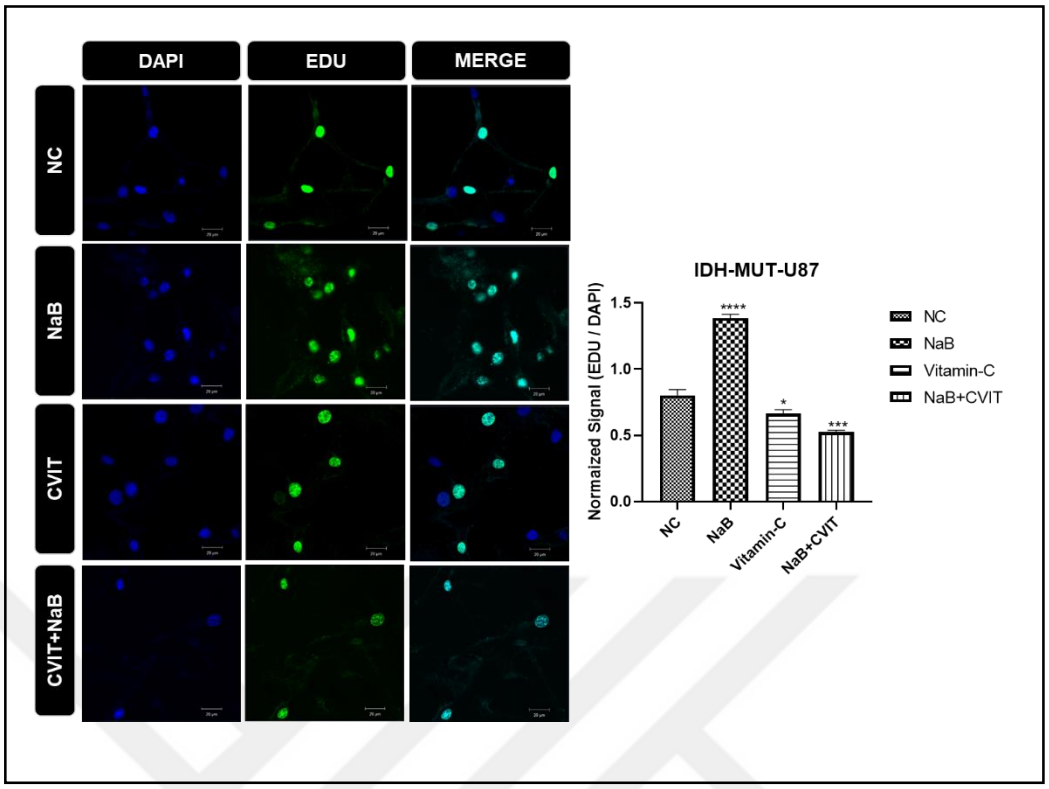

Figure 3.3.6. EDU assay result for IDH-MUT-U87 cell line. EDU green; dividing cell, blue; DAPI staining.

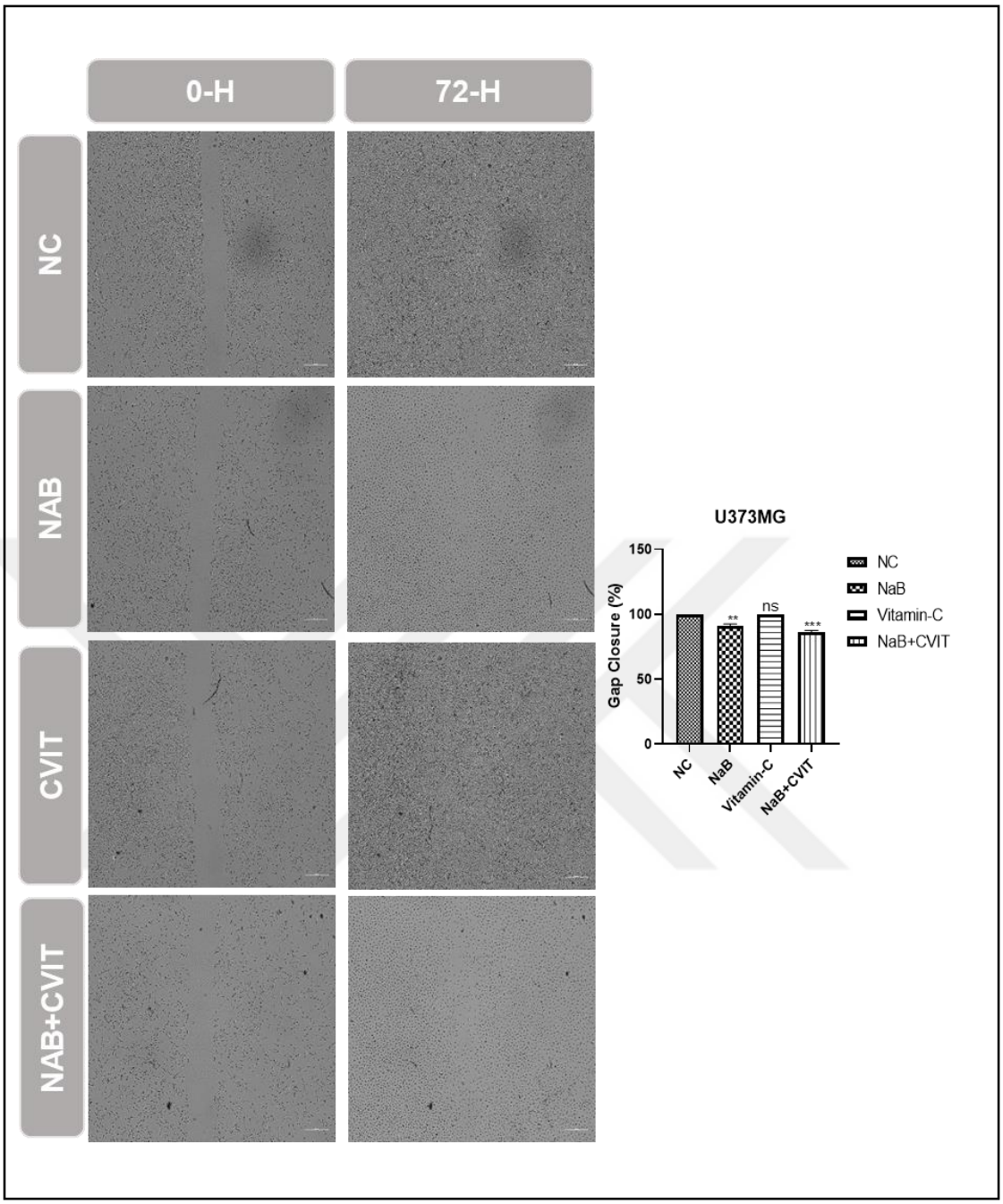

Figure 3.3.7. Representative results of effects of the various compound on the migration capacity of the U373MG cells at 72 hours.

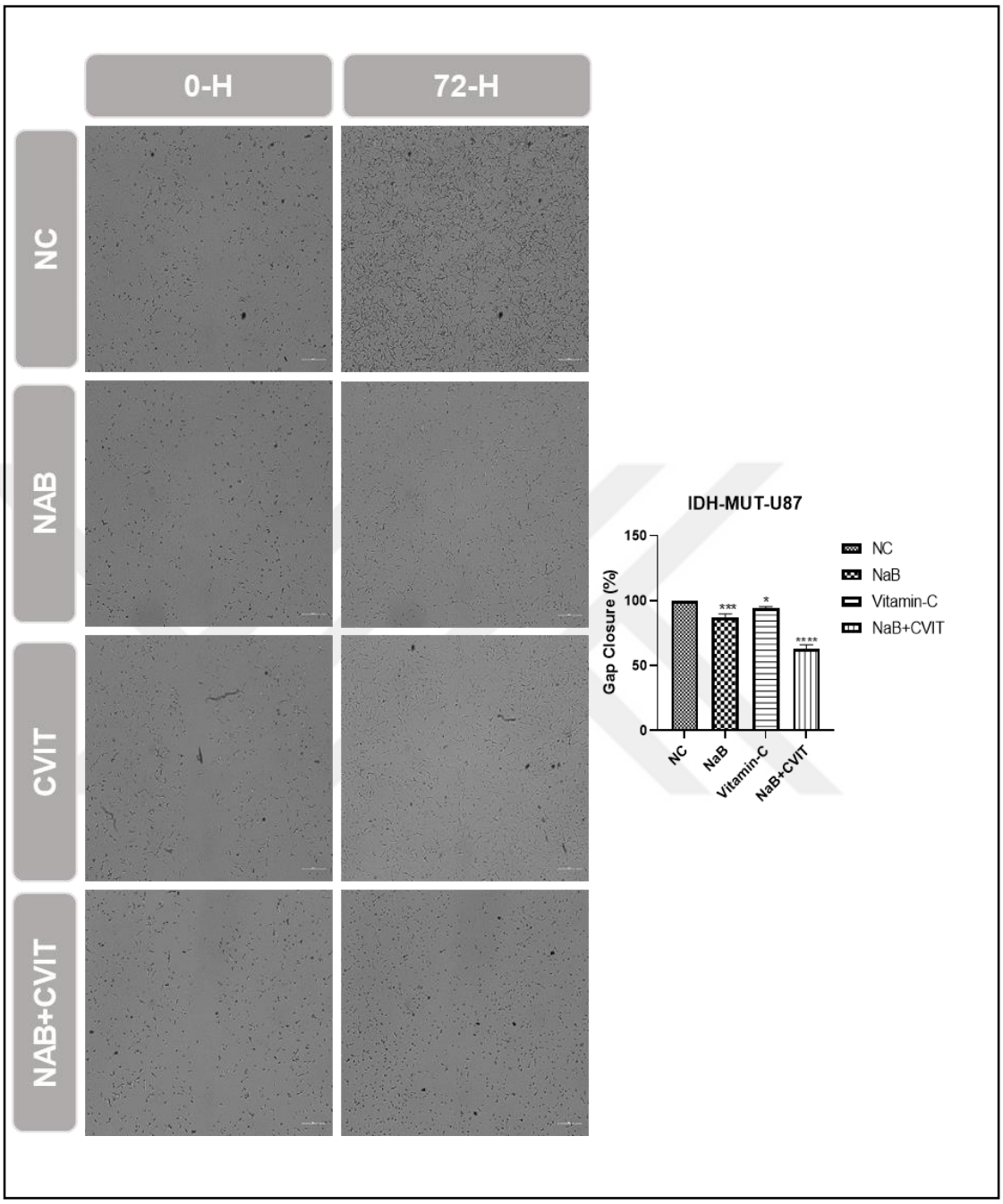

Figure 3.3.8. Representative results of effects of the various compound on the migration capacity of the IDH-MUT-U87 cells at 72 hours.

3.4.THE EFFECT OF NaB AND ITS COMBINATON WITH VITAMIN-C ON AUTOPHAGY CELL DEATH MECHANISM

To investigate whether IDH-MUT-U87 and U373MG cells could up-regulate autophagy to meet an elevated need for biomaterial, gene expression levels of the two key factors in autophagy (ATG-5 and LC3A/B) were analyzed by ICC experiment. As seen in Figure 3.4.3 and Figure 3.4.4, there was an increase in the LC3A/B genes for all treatment in both cancer cell lines. ATG5 genes was significantly increased in IDH-MUT-U87 and U373MG cell lines for each treatment except combination of Vitamin-C and NaB in U373MG cancer cells.

According to real-time PCR results, ATG-5, BECLIN, and LC3A genes was significantly decreased in the opposite way of the ICC experiment. Also, ATG5 genes was clearly increased NaB (≈1.43 fold) and their Vitamin-C combination (≈1.34 fold) and LC3A genes was upregulated in combination treatments (≈1.28 fold) for IDH-MUT-U87 cell lines.

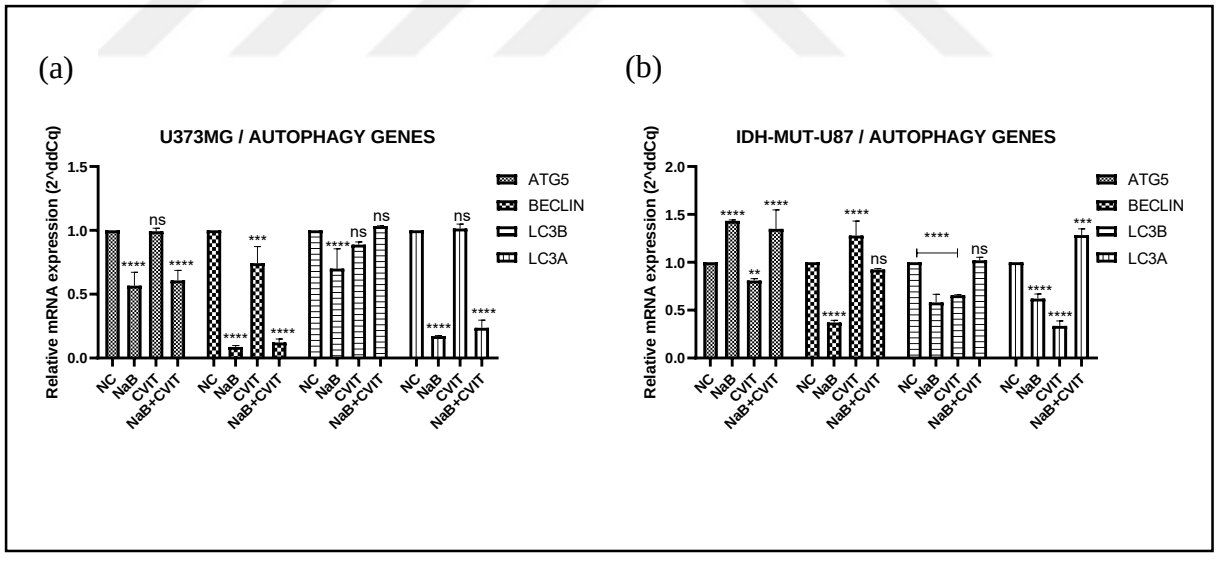

Figure 3.4.1. Autophagy genes of mRNA expression in (a) U373MG and (b) IDH-MUT-U87 cells after 72 h.

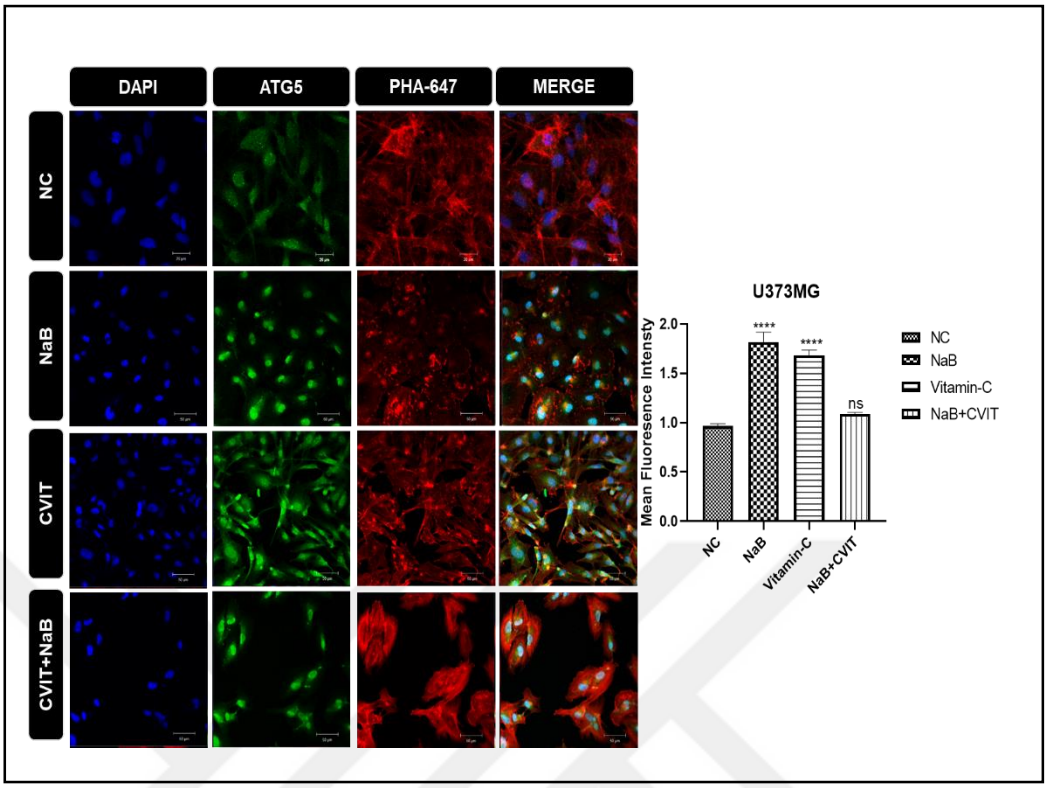

Figure 3.4.2. Representative result of ATG5 gene expression profiles in U373MG cells after 72 h. (green color; ATG5 expression, blue color; DAPI staining, red color; actin.

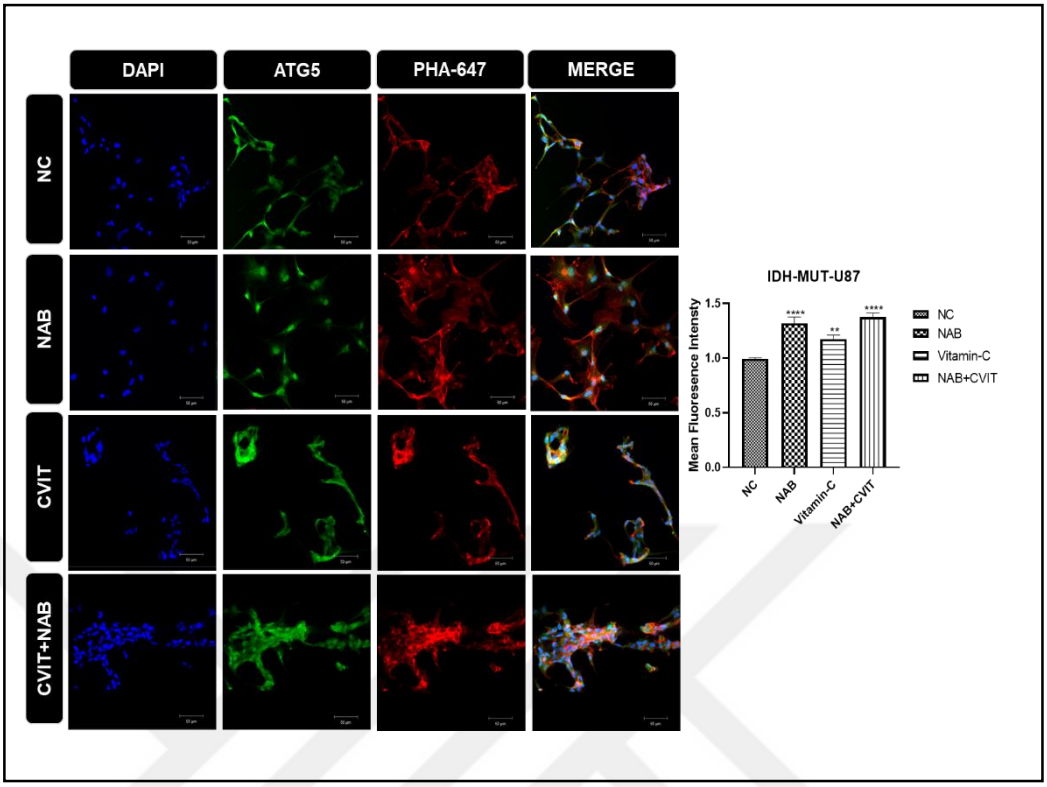

Figure 3.4.3 Representative result of ATG5 gene expression profiles in IDH-MUT-U87 cells after 72 h. (green color; ATG5 expression, blue color; DAPI staining, red color; actin.

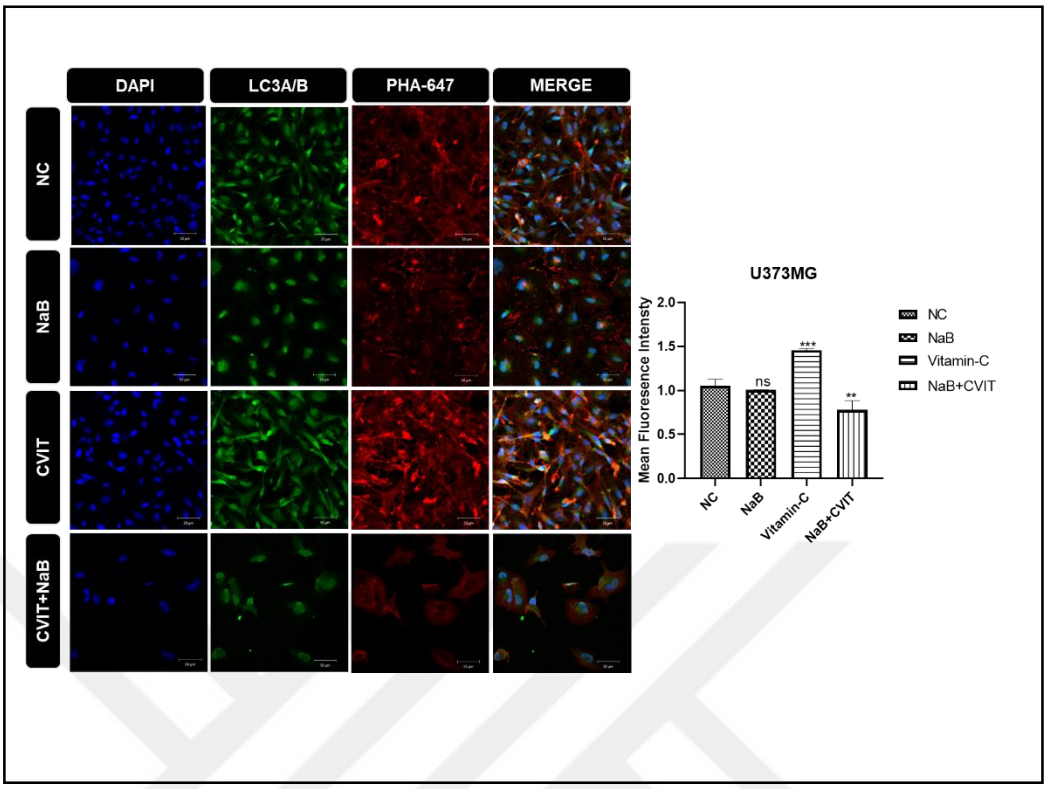

Figure 3.4.4. Representative result of LC3A/B gene expression profiles in U373MG cells after 72 h. (green color; ATG5 expression, blue color; DAPI staining, red color; actin.

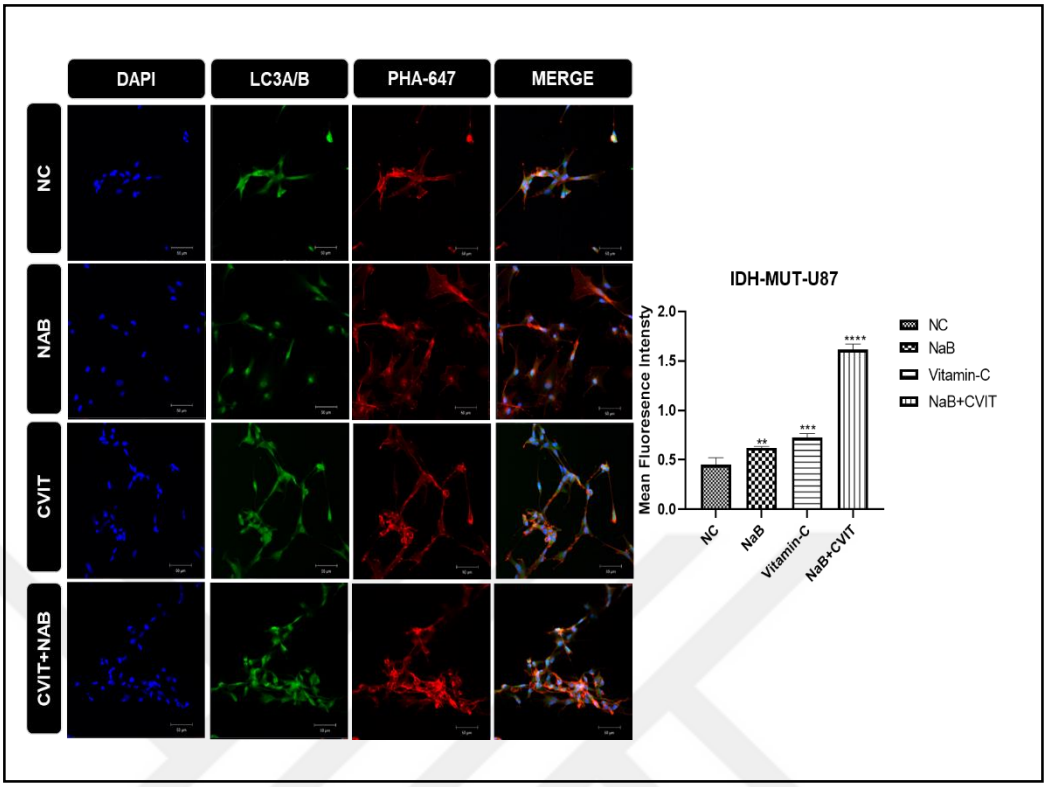

Figure 3.4.5. Representative result of LC3A/B gene expression profiles in IDH-MUT-U87 cells after 72 h. (green color; ATG5 expression, blue color; DAPI staining, red color; actin.

3.5.THE EFFECT OF NaB AND ITS COMBINATON WITH VITAMIN-C ON DNA DAMAGE MECHANISM

The expression levels of key genes of the DNA damage signalling pathway were investigated by NaB, vitamin-c alone and their combination on IDH-MUT-U87 and U373MG cancer cells. According to real-time PCR results, ATR genes of DNA damage sensor genes was significantly increased in alone NaB and their Vitamin-C combination for both cancer cells. ATM gene expression levels was upregulated ≈2.00 fold for NaB treatments and ≈2.05 fold for their Vitamin-C combination in U373MG cells. Also, these genes were significantly elevated NaB (≈3.6 fold) and NaB+VitC (≈8.51 fold) treatments in IDH-MUT-U87 cells. Essential DNA repair mechanism gene of Poly (ADP-ribose) polymerase 1 (PARP1) was significantly decreased combination of Vitamin-C and NaB both IDH-MUT-U87 (≈0.11 fold) and U373MG (≈0.18 fold) cells. Furthermore, according to the results of the ICC assay, the expression of the PARP1 gene was clearly decreased in all treatments for both cancer cells.

The effect of NaB and Vitamin-C treatments on ROS levels of both cancer cell lines was observed by DCFA kit assay. ROS levels was significantly increased exposing NaB and its combination with Vitamin-C combination for U373MG cell lines (Figure 3.5.4). In addition, in IDH-MUT-U87 cells, ROS levels was clearly elevated in treatments of Vitamin-C and its NaB combination as seen in Figure 3.5.5.

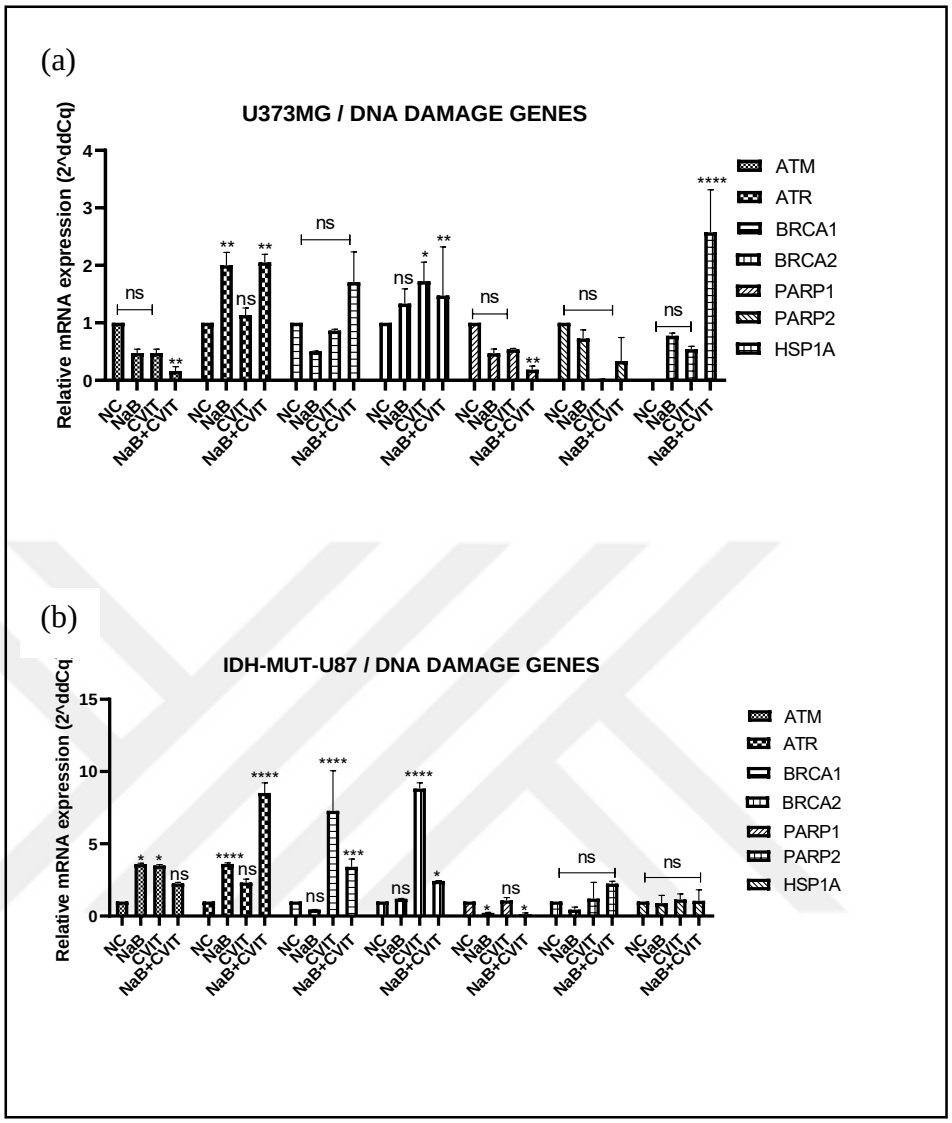

Figure 3.5.1. Representative graph DNA damage genes expression profiles in (a) U373MG and (b) IDH-MUT-U87 cells after 72 h.

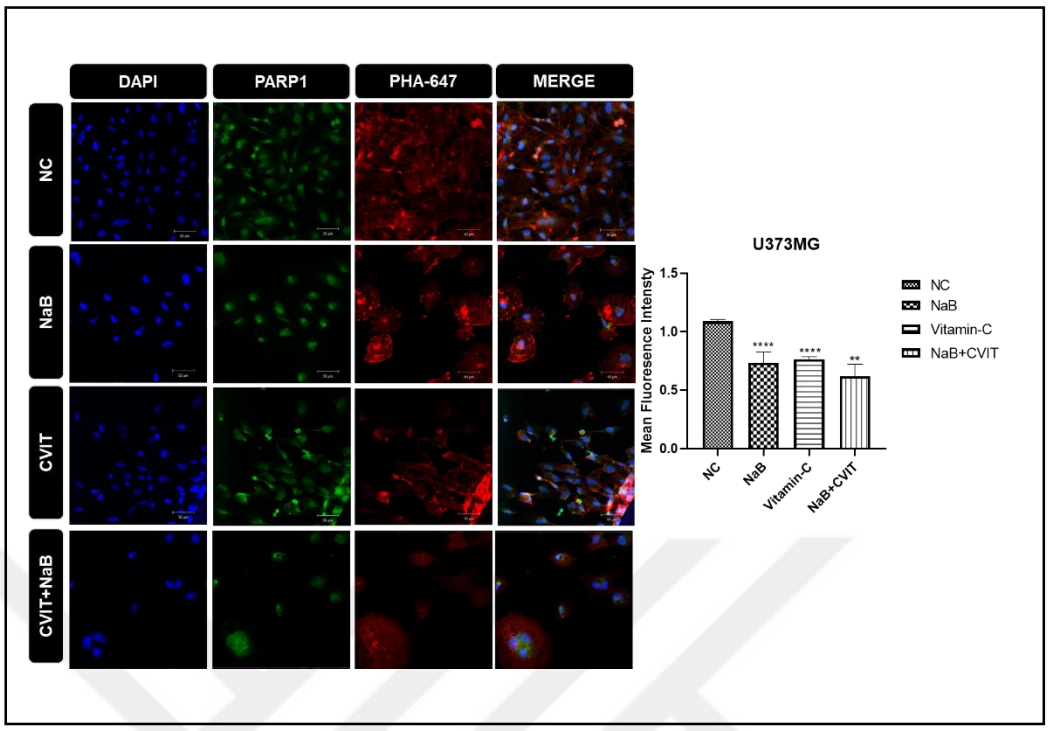

Figure 3.5.2 Representative result of PARP1 gene expression profiles in U373MG cells after 72 h. (green color; ATG5 expression, blue color; DAPI staining, red color; actin.

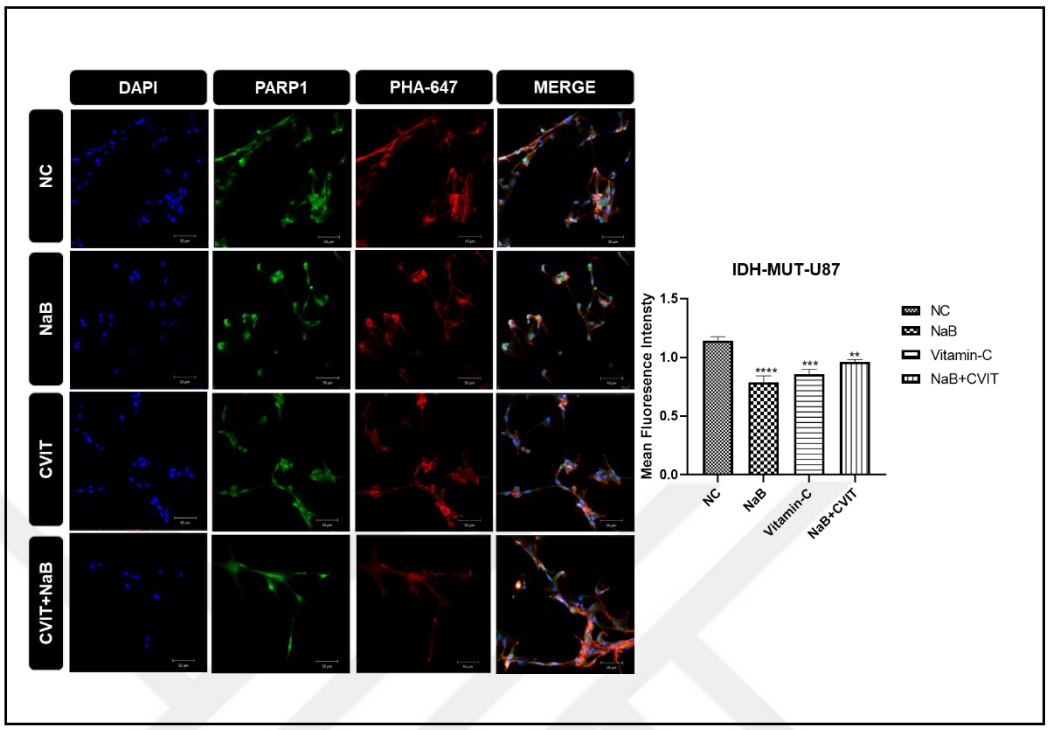

Figure 3.5.3 Representative result of PARP1 gene expression profiles in IDH-MUT-U87 cells after 72 h. (green color; ATG5 expression, blue color; DAPI staining, red color; actin.

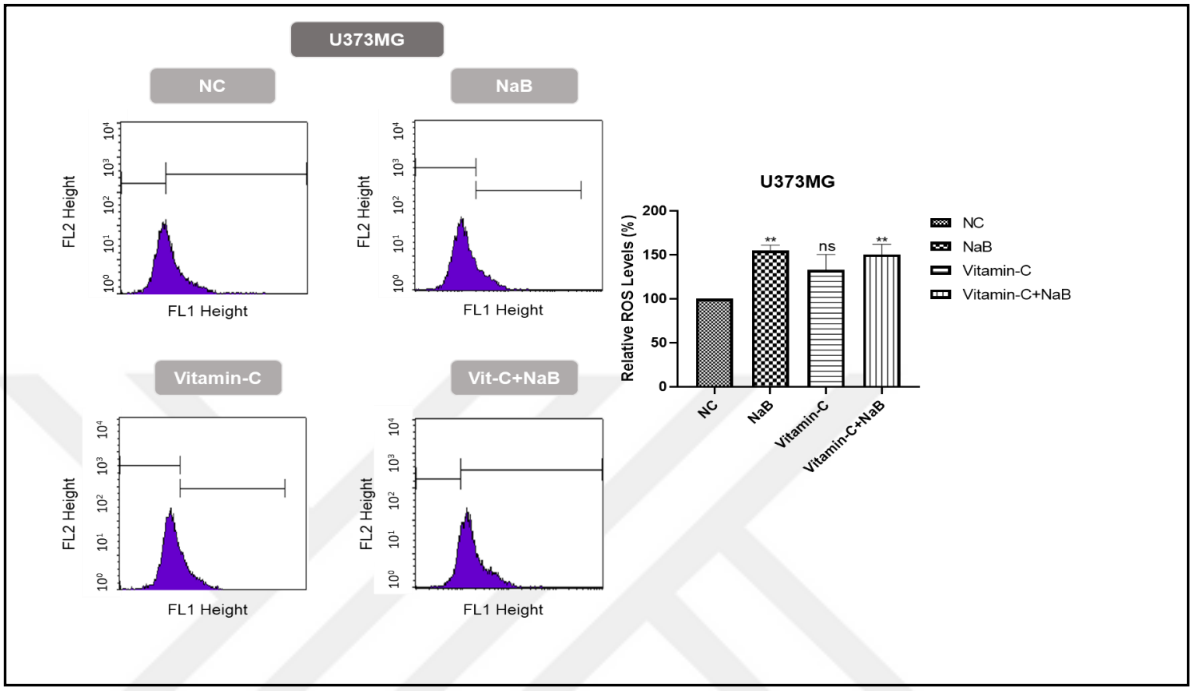

Figure 3.5.4 Representative result of ROS levels in U373MG cells by DCFA/H2DCFDA assay.

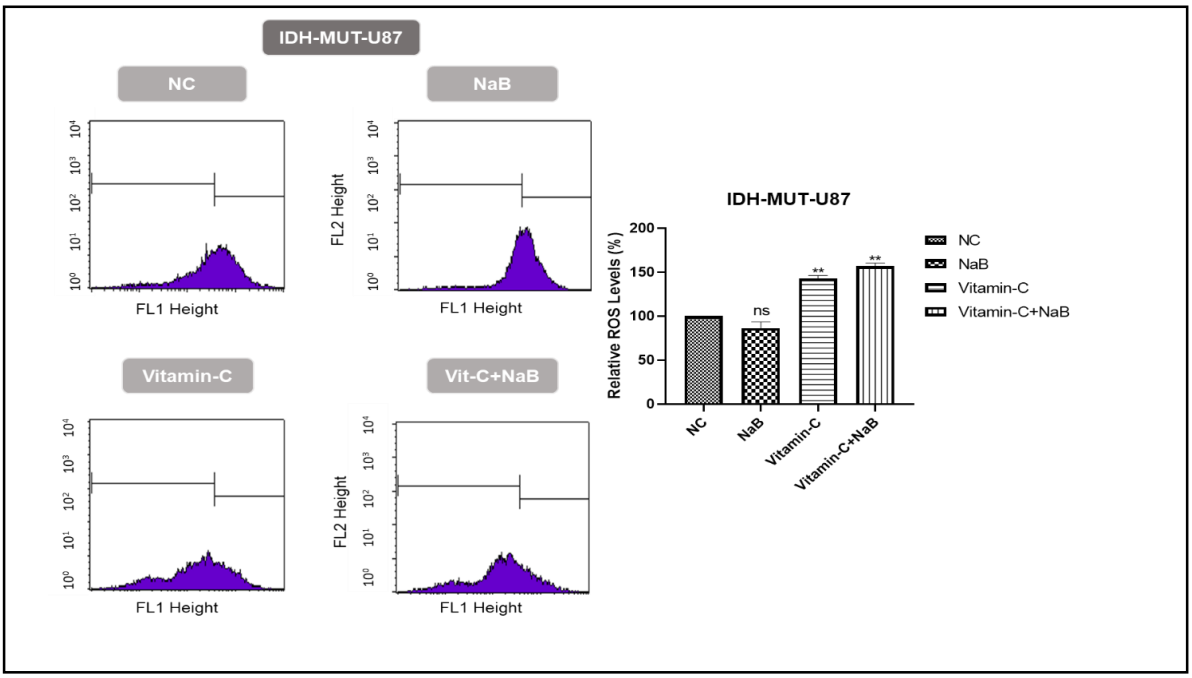

Figure 3.5.5 Representative result of ROS levels in IDH-MUT-U87 cells by DCFA/H2DCFDA assay.

4. DISCUSSION

Cancer, which is an illness caused by pathophysiological changes in the biological process of proliferation of cells, has become second most fatal disease that kills a lot of people every year all around the world [4]. According to GLOBACAN 2020, there are approximately 10 million cancer-related deaths per year and also 28.4 million new cases will be diagnosed worldwide by 2040 [126,127]. Therefore, demand and requirement for developing more effective and targeted medications for the treatment of various cancers has been stronger than ever [128–130].

Despite of the differences in the incidence of distinct forms of brain tumors based on race, sex, and other demographic factors, all brain tumors types have severe mortality. For example, although having a relatively low incidence, glioblastoma is one of the top 10 leading causes-of-death related to cancer [131]. In addition, nearly 40 percent of all malignant primary brain tumors are glioblastomas [132]. Numerous environmental and lifestyle factors, such as a number of vocations, spectrum of carcinogen exposure from the environment, and nutrition, have been linked to an elevated risk of glioma, while prior radiotherapy treatment is a categorically linked risk factor that predisposes to this type of brain tumor [133]. On the other hand, GBMs are tumors that are resistant to chemotherapy and there are unfortunately only few treatment options. The current status quo in the clinical outcome for GMB is that therapy improves patients' overall survival (OS), but neither cures nor prevents recurrences. Numerous studies have shown that DNA repair mechanisms including, Base excision repair (BER), DNA mismatch repair (MMR) and MGMT promote resistance in gliomas by reversing chemotherapy-induced damage [85,122]. Therefore, inhibition of MGMT has appealed interest as primary treatment strategy for overcoming resistance in GBMs.

Boron, which has a broad and significant physiological role in human body in terms of its gate-keeper role in hormonal and overall metabolism, is recognized as one of the vital nutraceuticals. Although its positive influence on various cellular processes are being increasingly reported in recent studies, it is not an essential nutritional element for humans. *In vitro*, animal, and human clinical studies have shown that boron is a natural element in nutrient quantities that benefits bone growth and normal central nervous system activity, makes hormone action easier, and is linked to a lower risk for some cancers. [134]. One of

the most recent proposed benefits of boron includes lowering the risk of certain forms of cancer [134–136]. Boron is abundant in Turkey and in this study, its use in combination with Vitamin-C was shown to have an anti-cancer effect in GBM cancer. This thesis focuses on the advantages of combining boron-based compounds with chemotherapy drugs such as Temozolomide and Olaparib, highlighting the richness of boron resources in Turkey, and conducting research on reducing the toxic effects of cisplatin through dosage reduction. This study is considered as a significant step towards developing a more effective and safer chemotherapy strategy.

Here, NaB, Vitamin-C, TMZ, olaparib and Vitamin-C either as single agents or in combination displayed cytotoxic effects in different concentration ranges on the malignant cell lines of IDH-MUT-U87, U373MG, but not on the normal human astrocytes (NHA cell line). There is only a mild difference in cytotoxicity between TMZ alone treatment and its combination with Vitamin C in the IDH-MU-U87 cells (Table 3.1). In addition, although the half maximal inhibitory concentration (IC50) of Olaparib has been reduced by a factor of two in the combination treatment of Olaparib with Vitamin-C both in the IDH-MUT-U87 and U373MG cell lines, the same combination revealed a toxic effect in the normal NHA astrocytes cell line. Remarkably, combination of Vitamin-C with NaB resulted in a 5 fold decrease in the NaB IC50 value obtained in the mono-treatments of NaB in the IDH-MUT-U87 cells. Also, NaB was shown to be effective in both IDH-MUT-U87 and U373MG cell lines as a single agent. Both NaB alone and its combination with Vitamin-C effectively kill cancer cells without damaging normal cells (NHA). Thus, either NaB as a mono-agent or its combination with Vitamin-C can be a beneficial therapeutic approach in the treatment of GBM patients. Also, Vitamin-C could be used as an adjunvant chemotherapy agent to reduce olaparib-related toxicity in patients receiving olaparib.

Vitamin-C and Olaparib combination is more effective compared to the combination of Vitamin C and TMZ, because Vitamin-C is cofactor of TET2 enzyme, this nutrient plays a modulatory role in the DNA methylation. Since, IDH mutations particularly impair TET enzymes, resulting in abnormal hypermethylation in the malignant GBM cells, combination of Vitamin-C with Olaparib could be eliciting a more potent response than the combination of Vitamin-C with TMZ due to the impact of Vitamin-C on the TET2 enzyme.

Apoptosis is a form of cellular death process that eliminates unneeded cells from the human body. It is regulated by intricate signaling networks comprised of several factors. Annexin-

V assay and real-time experiments were conducted to evaluate the effect of the drug combinations-of-interest on the activation of apoptotic mechanisms. In the study it was discovered that as a potential new class of anti-cancer agents, the combination of Vitamin-C and NaB stimulate some apoptosis related genes and suppress the proliferation of the IDH-MUT-U87 cancer cells and promote cellular death. According to the results of Annexin V assay, combination of NaB and Vitamin-C lead to a significant stimulation of apoptosis accompanied by a rise in the levels of effector caspase 7, initiator caspase 8, TP53 tumor suppressor genes in IDH-MUT-U87 cells. Although a similarly potent stimulation of apoptosis did not take place in the U373MG cells, caspase 8 expression levels was significantly increased and TP-53 gene expression levels was decreased in all treatments.

A possible reason of this discrepancy might be explained by the differential responses of the two cell lines to NaB, Vitamin-C and the combination of both due to the differences in their genetic composition, growth rate, and mutational burden. For example, one study has shown mutation of PTEN, TERT, TP53, P16/CDKN2A in U373MG while mutations of NF1, TERT and PTEN in U87MG. Also, in GBM TP53 mutation is oncogenic, but PTEN genes must also be altered to promote the GBM progress [137]. GBM cells with TP53 mutation undergo reduced levels of apoptosis [138], are more invasive [139] and possess a higher proliferative index [140].

In terms of alterations in cell cycle properties, the decrease in the G1 population of the IDH-MUT-U87 cells were followed up by examining the levels of the key regulators of G1-S such as CDK4, CDK6, Kİ67, PCNA, Survivin genes, all of which are well known for their role both in proliferation and migration. The mRNA levels of all these genes were significantly reduced in support of the cell cycle phenotype and results of the scratch assay, where migratory properties of the cells exposed to combination of NaB and Vitamin-C were hindered.

In IDH-MUT-U87 cells there were 14.2% G2/M phase alterations in response to NaB and Vitamin as seen in Figure 3.3.2. Although, the increase in G2 phase population of IDH-MUT-U87 cells could be explained by a concomitant decrease in CDK1 levels upon the treatment with combination-of-interest. In the U373MG line the same combination elicited 25 % changes in G1 and G1/S transition (Figure 3.3.1).

Regarding the expression levels of cell cycle regulators of the G1-S and S phase there was surprisingly a rise in the CDK2 levels while U373MG cells appeared to have undergone an S phase arrest. This discrepancy will be addressed in future work where both levels and activity of CDK2 kinase will be analyzed in Western Blot analyses using total and phospho-specific antibodies as a read-out for CDK2 catalytic activity. One possibility could be that although CDK2 mRNA levels may have increased, the corresponding protein could be inhibited via well-known post-translational modifications in control of CDK2 activity such as activating Tyr164 and/or inhibitory Thr14/15 phosphorylations. For example, a decrease in Tyr164 phosphorylation by CDK Activating Kinase (CAK) or an increase in Thr14/15 by Wee1-associated kinase could have blocked CDK2 activity resulting in the observed S phase arrest.

Besides, cell migratory capacities were decreased in the combination treatments with NaB and Vitamin-C, also to changes in cell size and shape as visualized by the light microscope in the routine culture of U373MG cell lines. Furthermore, the cell cycle regulator CDKN1A is largely responsible for regulating cell growth and proliferation [141]. This study has shown that this gene is significantly increased in the treatment with the combination of Vitamin-C and NaB in both cancer cell lines. Nonetheless, the levels of Ki67, PCNA, Surviving genes were significantly decreased when the U373MG cells were exposed to the Vitamin-C and NaB combination. Futhermore, considering that there is a close relationship between DNA synthesis and cell proliferation, the amount of DNA synthesis was determined by EDU staining. In all treatment groups in both cell lines, DNA synthesis was decreased compared to negative control and nuclear size and shape was altered (Figure 3.3.6). Overall findings, findings from the gene expression analyses, DNA synthesis, cell cycle assay, and scratch assay point to a significantly decreasing effect by the combinations-of-interest, revealing a cytotoxic impact in the IDH-MUT-U87 and U373MG cancer cell lines.

A biological process whereby parts of cells and damaged organelles are digested during lysosomal activity is known as autophagy, that is a primary cell catabolic mechanism [142]. By enabling cells to adjust to challenging situations, autophagy helps to maintain cellular homeostasis and prevents cellular damage. ATG5 is essential for controlling when autophagosomes develop [143] and Structure-preserving proteins called LC3A and LC3B are found in the membranes of phagophores and autophagosomes [144]. Moreover, in both IDH-MUT-U87 and U373MG cells, ATG5 and LC3B expression levels was determined at

level of protein and mRNA expression. Mechanistically, this study reports that induced growth inhibition upon treatments with the combination of NaB and Vitamin-C promotes an autophagy response via consistent upregulation of ATG5 and LC3A mRNA and protein levels in the IDH-MUT-U87 cancer cells. On the other hand, although mRNA levels of ATG5, BECLIN and LC3A/B decreased in U373MG cells, ATG5 protein expression was clearly increased. This discrepancy can be explained by the fact that under some circumstances the protein level can be elevated due to increased stability despite a decrease in the mRNA levels of the same gene. While it is common to assume that a reduction in mRNA would be reflected as a decline in the protein levels, there are a number of exceptional circumstances protein levels might change inconsistently with respect to the mRNA level due to altered protein turnover properties. For example, proteins might undergo alteration after translation that affects their activity, or destruction. In even with a drop in mRNA levels, certain changes, such as phosphorylation and ubiquitination, might stop protein from degrading. According to our findings, the combination of NaB and Vitamin-C treatment induced autophagy in the IDH-MUT-U87 cancer cells, but increased protein levels of atg5 gene alone may not be sufficient to induce autophagy mechanisms.

The targeting of DDR-dependent mechanisms as novel approach in the treatment of GBM has increased in significance in the recent years [145]. Therefore, expression of the key factors of the DDR pathway, including ATM, ATR, BRCA1/2, HSP1A, and PARP1/2 were investigated and ROS levels were measured in both IDH-MUT-U87 and U374MG cell lines in response to the combinations-of-interest. We found that ROS levels are increased upon dual treatment with NaB and Vitamin-C. Due to their capacity to promote apoptosis, enhanced ROS can also be used as a powerful method to eliminate cancer cells [146,147]. Furthermore, it is hypothesized that cancer cells may be more vulnerable to the build-up of ROS than normal cells, making exogenous increase in ROS levels a suitable and selective point of intervention that aims to enhance oxidative stress as an anticancer approach targeting cancer cells while sparing normal cells [148–150].

Another noteworthy finding of this study is that PARP1 protein levels were significantly decreased in the combination treatment of NaB and Vitamin-C in both cancer cells. The most well-known function of PARP1 is in DNA repair pathways [151]. Many studies have reported that PARP1 levels are also hyper-induced in other cancer types such as hepatocellular carcinoma, ovarian and breast cancer [152]. In terms of GBM, it was

discovered that higher PARP-1 expression levels were detected in GBM samples than those in normal donor tissues [153,154]. These results collectively imply that inhibiting PARP activity may be helpful in the treatment of tumors.

5. CONCLUSION

Findings in this study suggest that combination of Vitamin-C and NaB is a promising novel therapeutic alternative for the treatment of GBM tumors. We have clearly demonstrated that the dual Vitamin-C and NaB combination induces apoptotic and autophagy pathways, inhibits cell migration, survival, and proliferation in the *in vitro* models of GBM, IDH-MUT-U87 and U373MG GBM cancer cell lines. Remarkably, these results show that while these compounds exhibit enhanced therapeutic effect, anti-tumorigenic and preventive role in GBM cancer in the tested cell lines, NaB and its combination with Vitamin-C did not exert harmful effects on the normal human astrocyte cells. In addition, combining Vitamin-C with an existing chemotherapeutic drug, Olaparib, displayed a synergistic effect in terms of cytotoxicity to malignant cells and it decreased IC50 value of Olaparib as a single agent by two fold, suggesting Vitamin-C as a safe adjuvant agent that can lower side effects of Olaparib. As a consequence, these results point to a potential advantage of utilizing combination of Vitamin-C with either NaB or Olaparib in the treatment of GBM tumors. Future studies should investigate the therapeutic potential of various dual or triple combinations of Vitamin-C with both NaB and Olaparib as well as other drug candidates being developed for the treatment of GBM both in the *in vitro* and *in vivo* models of this life-threatening disease. In future perspectives, in vivo studies will be carried out and the working mechanism will be examined more. Finally, although more work is needed to create safer and more effective cancer drugs, this study demonstrated that Nab and Vitamin-C substances and their combinations have the potential to be used in future cancer treatments.

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