COMBINATORY EFFECT OF 3-BROMOPYRUVIC ACID AND CURCUMIN ON COLORECTAL CANCER

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COMBINATORY EFFECT OF 3-BROMOPYRUVIC ACID AND CURCUMIN ON COLORECTAL CANCER

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ABSTRACT

COMBINATORY EFFECT OF 3-BROMOPYRUVIC ACID AND CURCUMIN ON COLORECTAL CANCER

Colorectal cancer (CRC) is the third common cancer type in both males and females in Turkey. One of the hallmarks of cancer states that cancer cells reprogram their energy metabolism, known as aerobic glycolysis, to fuel cell division and growth by producing lactate even in the presence of oxygen. Therefore, regulation of glycolysis plays a crucial role in cancer therapies. 3-bromopyruvate (3BP) causes the suppression of the glycolytic enzyme hexokinase II (HKII), results in low levels of ATP and cell death in cells. Curcumin (CUR) affects different types of cancers such as liver, skin, breast, kidney, hepatocellular carcinoma, and colorectal through apoptotic cell death. In this study, we aimed to reveal the effects of 3BP and CUR combination treatment (COT) on cellular, glycolytic, and mitochondrial metabolism in HCT116, colorectal cancer cell line. First, we revealed COT inhibits cell proliferation by 32 percent compared to each treatment alone. Cell death mechanism analysis showed that COT doubled late apoptotic cell number. The analysis with 2',7'–dichlorofluorescein diacetate fluorogenic dye to measure the generation of reactive oxygen species increased upon COT by 90 percent. Then, mitochondrial membrane potential by Rhodamine 123 dye and mitochondrial mass by Mitotracker Green FM dye were analyzed which was found as decreased by COT treatment to 35 and 36 percent, respectively. Also, alterations in protein expressions of mitochondrial OXPHOS complexes were determined by immunoblotting which revealed that Complex III (cytochrome bc1) and Complex II (succinate dehydrogenase) protein expression levels were decreased by 21 and 32 percent. Lastly, glycolytic metabolism was investigated by analyzing LDH-A protein expressions by immunoblotting which was decreased LDH-A levels to 36 percent upon COT. According to the obtained results, 3BP and CUR combination might be a potential therapeutic agent for colorectal cancer by limiting both glycolysis and OXPHOS bioenergetic pathways.

ÖZET

3-BROMOPİRÜVİK ASİT VE KURKUMİN'İN KOLOREKTAL KANSER ÜZERİNDEKİ KOMBİNASYON ETKİSİ

Kolorektal kanser (CRC), Türkiye'de hem erkeklerde hem de kadınlarda görülen üçüncü yaygın kanser türüdür. Kanserin ayırt edici özelliklerinden biri, kanser hücrelerinin, aerobik glikoliz olarak bilinen enerji metabolizmalarını, oksijen varlığında bile laktat üreterek yakıt hücresi bölünmesini ve büyümesini yeniden programladığını belirtir. Bu nedenle, glikolizin düzenlenmesi kanser tedavilerinde çok önemli bir rol oynar. 3-bromopiruvat (3BP), glikolitik enzim hekzokinaz II'nin (HKII) inhibisyonuna neden olur ve böylelikle hücrelerde düşük ATP seviyeleri ve hücre ölümü ile sonuçlanır. Kurkumin (CUR), apoptotik hücre ölümü yoluyla karaciğer, deri, meme, böbrek, hepatoselüler karsinom ve kolorektal gibi farklı kanser türlerini etkiler. Bu çalışmada, HCT116, kolorektal kanser hücre hattında 3BP ve CUR kombinasyon tedavisinin (COT) hücresel, glikolitik ve mitokondriyal metabolizma üzerindeki etkilerini ortaya koymayı amaçladık. İlk olarak, COT'un diğer tedavilere kıyasla hücre çoğalmasını yüzde 32 oranında engellediğini ortaya çıkardık. Hücre ölüm mekanizması analizi, COT'nin hücrelerde yüzde 99 geç apoptoza yol açtığını gösterdi. Reaktif oksijen türlerinin üretim hızını ölçmek için 2', 7'-dikloroflüoresein diasetat florojenik boya ile yapılan analiz, COT üzerinde yüzde 90 oranında artmıştır. Daha sonra Rhodamine 123 boyası ile mitokondriyal membran potansiyeli ve Mitotracker Green FM boyası ile mitokondriyal kütle analiz edildi ve COT tedavisi ile sırasıyla yüzde 35 ve 36'ya düşürüldü. Ayrıca, mitokondriyal OXPHOS komplekslerinindeki değişiklikler, Kompleks III (sitokrom bc1) ve Kompleks II (süksinat dehidrojenaz) protein ekspresyon seviyelerinin yüzde 21 ve 32 oranında azaldığını gösteren immünoblotlama ile belirlendi. Son olarak glikolitik metabolizma, LDH-A protein ekspresyonlarının COT ile yüzde 36'ya düşürüldüğü immünoblotlama ile analiz edilerek araştırıldı. Elde edilen sonuçlara göre, 3BP ve CUR kombinasyonu, hem glikolizi hem de OXPHOS biyoenerjetik yollarını sınırlandırarak kolorektal kanser için potansiyel bir terapötik olabilir.

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LIST OF SYMBOLS/ABBREVIATIONS

μM	Micromolar
2-DE	2-Dimensional gel electrophoresis
3BP	3-Bromopyruvic acid
Acetyl-CoA	Acetyl coenzyme A
ACF	Aberrant crypt foci
ADP	Adenosine diphosphate
AIF	Apoptosis inducing factor
AJCC	American joint committee on cancer
ATP	Adenosine triphosphate
CAMs	Cell-cell adhesion molecules
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CT	Computed tomographic colonography
CUR	Curcumin
DCBE	Double-contrast barium enema
DCF	2’,7’-Dichlorofluorescein
DCFDA	2’,7’-Dichlorofluorescein diacetate
DIC	Deacetylation inhibitor cocktail
DMEM	Dulbecco’s modified eagle’s medium
DNA	Deoxyribonucleic acid
ECAR	Extracellular acidification rates
ECM	Extracellular matrix
EMT	Epithelial–mesenchymal transition
ER	Endoplasmic reticulum
ETC	Electron transport chain
FADH2	Flavin adenine dinucleotide
FDA	Food and drug administration
FOBT	Fecal occult blood test
FS	Flexible sigmoidoscopy colonoscopy

GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gFOBT	Guaiac-based fecal occult blood test
GLOBOCAN	Global cancer incidence, mortality and prevalence
GLUT1	Glucose transporter 1
GS	Growth signals
GSH	Glutathione
H2O2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HCT116	Human colorectal carcinoma cell line
HIF1α	Hypoxia-inducible factor 1 alpha
HKII	Hexokinase II
JNK	c-jun N-terminal kinase
iFOBT	Immunochemical fecal occult blood test
IMM	Inner mitochondrial membrane
LDH-A	Lactate dehydrogenase A
M	Molar
MAPK	p38 mitogen activated kinase
MCTs	Mono-carboxylate transporters
ml	Milliliter
mtDNA	Mitochondrial DNA
NADH	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA
O2•-	Superoxide anion radical
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PBS	Phosphate buffered saline
PDC	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PI	Propidium iodide
PMSF	Phenylmethanesulfonylfluoride
PPP	Pentose phosphate pathway
pRb	Retinoblastoma protein
PS	Phosphatidylserine

PVDF	Immune-blot polyvinylidene difluoride
RIPA	Radio-immunoprecipitation assay
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RS	Reactive species
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TCA	Tricarboxylic acid cycle
TNF-α	Tumor necrosis factor alpha
TNM	Presence of tumor (T), local lymph node metastases (N), distant metastasis (M)
TP53	Tumor protein 53
TSP-1	Thrombospondin 1
TÜİK	Türkiye İstatistik Kurumu
UCP2	Mitochondrial uncoupling protein-2
UICC	The International Association of Cancer
VDAC	Voltage-dependent anion channel
VEGF-A	Vascular endothelial growth factor-A

1. INTRODUCTION

1.1. CANCER

Cancer is one of the most common and frequently diagnosed diseases in the world and its incidence and mortality rates are accelerating rapidly. The growing and aging population of the world causes cancer to be seen more, and nevertheless, the presence of increased risk factors for instance economically underdeveloped countries, smoking, obesity, inactive lifestyle, and environmental pollution arising from urbanization increase the prevalence of cancer [1]. Cancer ranks second among the causes of death both in our country and around the world. According to the data obtained, it is determined that approximately one in six deaths globally and one in five deaths in our country are due to cancer [2,3]. According to the Turkey statistics agency data (TÜİK), statistically, the incidence of cancer in men was found as decreased, while there was not any change in female cancer incidence when the data of the last five years are analyzed. The first five common types of cancer seen in Turkey are similar to the pattern in other developed countries around the world such as America and countries in Europe. Trachea, bronchus and lung, prostate, and colorectal cancer types in men and breast, thyroid, and colorectal cancer types in women are ranked first in Turkey (Figure 1.1). Statistics in 2015, 97.830 men and 69.633 women in Turkey were estimated to be diagnosed with cancer in 2015 [4].

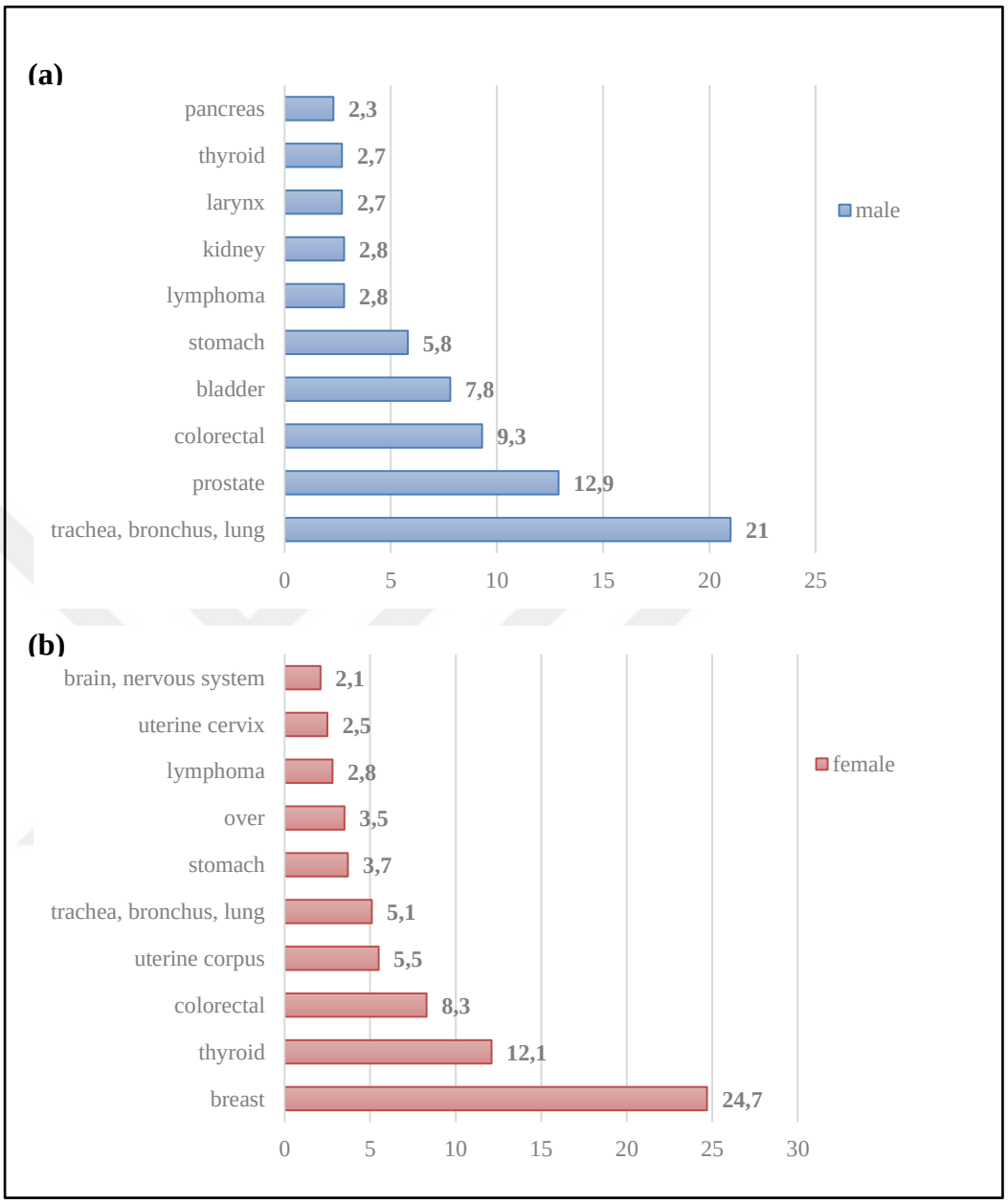

Figure 1.1. Percentage distribution of common cancer types of all age groups. (a) in males, (b) in females [4].

1.1.1. Hallmarks of Cancer

Cancer has been indicated to be a complex disease *in vivo* and *in vitro* laboratory and clinical studies. Considering recent studies to simplify the underlying causes of this complex disease,

some molecular, biochemical, and cellular parameters are common features shared by most or almost all cancer cells. There are 10 basic alterations that a cancer cell has to produce malignant growth: self-sufficiency in growth signals, tissue invasion, and metastasis, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, evading immune destruction, reprogramming of energy metabolism, evading apoptosis, genomic instability, and tumor-promoting inflammation (Figure 1.2) [5].

Advanced and sustainable cell proliferation is one of the key hallmarks of cancer cells. Normal cells maintain cell growth and division by controlling the generation and release of mitogenic growth signals (GS), thereby preserving the normal cell structure and function by maintaining the homeostasis of cell number. Once these mitogenic growth factors are released and transmitted by the transmembrane receptors into the cell, the growth factors bind to distinctive signal molecules such as cell-cell adhesion molecules and extracellular matrix (ECM) components, thereby stimulating normal cells within the quiescent state. however, cancer cells produce their growth signals, disrupting the regulation of this growth signal-dependent control mechanism. cancer cells can acquire some alternative ways to gain sustainable cell division capacity. one of these alternatives is that they synthesize growth factor ligands themselves. thus, with the expression of cognate receptors, it leads to proliferative stimulation in cancer cells. Another alternative is that cancer cells induce normal cells in the tumor stroma by sending them specific signals to supply growth factors to the cancer cells [5,6].

Another capability of cancer cells is that they can evade growth suppressors. normal cells control the cellular proliferation by receiving antiproliferative signals from the environment, allowing the cell to remain in a quiescence state, and homeostasis is also maintained. cancer cells, on the other hand, become insensitive to anti-growth signals, by suppressing tumor suppressor genes and bypassing some cell cycle checkpoints. cells monitor their external environment and decide whether they will proliferate, be in a quiescent state, activate apoptosis or postmitotic state based on the signals received from the environment. One of the proteins that is crucial for making this decision and serving as a gatekeeper in the cell cycle is retinoblastoma (pRb) protein and the other one is the tumor protein 53 (TP53). While the pRb protein transduces anti-growth signals, the TP53 protein senses stress and abnormal events within the cell. Defects in pRb protein and cell cycle are seen in most types of cancer cells which promotes persistent cell proliferation [5,7].

The proliferation of cancer cell populations is related to proliferation rate of cells and to apoptosis. As stated in cell death mechanism studies, *in vivo* mouse models and *in vitro* studies, almost all cancer types have been reported to have acquired resistance to apoptosis. This resistance in cancer cells occurs in various ways. The most common one of these strategies arises from mutations which are formed in the TP53 gene. As a result of functional loss in TP53 protein, cancer cells can avoid sensing critical damages in the environment and thus limit or prevent apoptosis [6].

Besides cell growth programs, cancer cells have unlimited replicative potential. In life cycle of normal cells have a program to prevent limitless proliferation. Thus, the cells acquired limited replicative potential which is called a Hayflick limit, and when a certain number exceeds the doubling number, they pass to the stage called senescence. In studies conducted, it was determined that most types of cancer cells were immortal when cultured and gained this feature during tumor progression *in vivo*. According to many studies, the unlimited division of cancer cells is mainly due to changes in telomere activity. In normal cells, telomeres consisting of multiple tandem hexanucleotide repeats are shortened in each cell division, causing the cell to age and die. Cancer cells, on the other hand, prevent the shortening by keeping the telomeres above the critical length due to the maintenance provided in the telomerase enzyme and thus gain replicative immortality feature [7].

Like normal cells, tumor cells need nutrients, oxygen, and detoxification of metabolic wastes. The process is called angiogenesis that provides all these needs of tumor cells with the formation of tumor-associated neovasculature. At the embryonic stage, new endothelial cells are formed and assembly into a tube called vasculogenesis. Also, new vessel formation except for existing vessels is called angiogenesis. Normal vasculature continues as quiescent after this stage. In adults, angiogenesis is active for a short and temporary period in physiological conditions such as wound healing. On the contrary, angiogenesis is constantly active to ensure tumor growth, sustain and preserve new vessel formation. Vascular endothelial growth factor-A (VEGF-A), an inducer, and thrombospondin 1 (TSP-1), an inhibitor, are suggested to be crucial in the mechanism of angiogenesis. Factors such as hypoxia and also oncogene signaling have been found to increase VEGF gene expression, which is responsible for important processes such as the forming of new blood vessels, and homeostasis of endothelial cells [5].

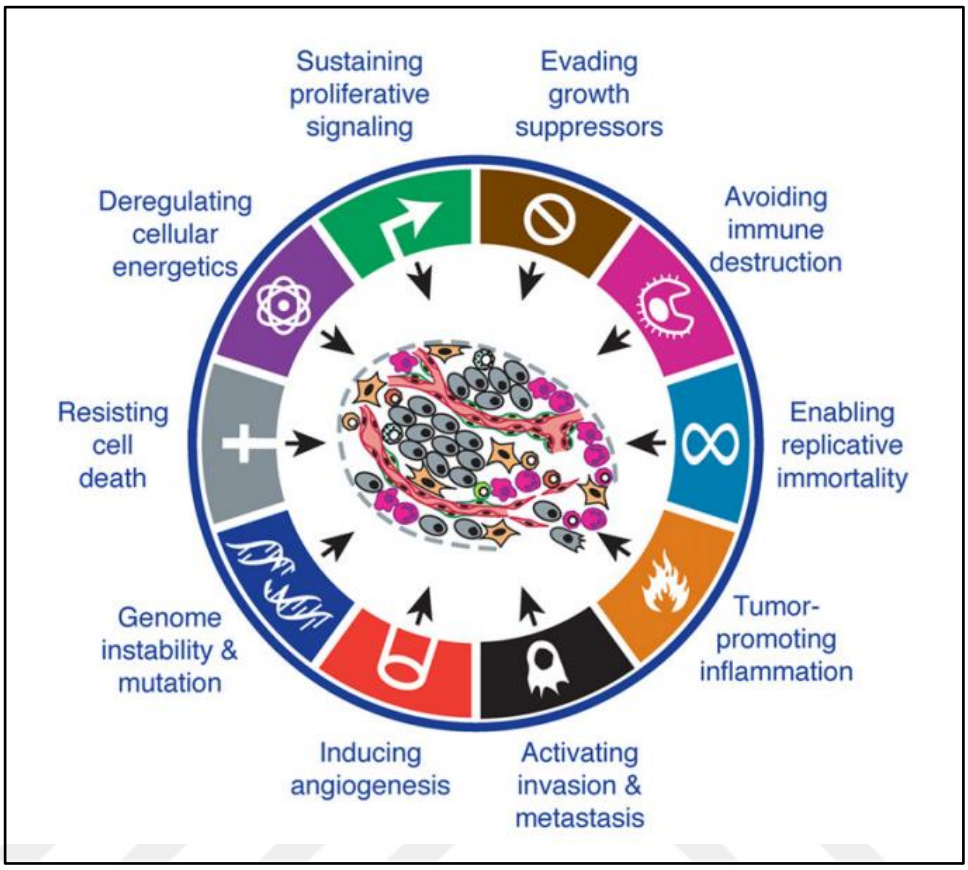

Figure 1.2. The ten hallmarks of cancer cells [7].

During the development of most types of tumor cells, primary tumor cells reveal new and precursor cells, invade nearby tissues and travel to new locations where they can establish new colonies. This process that tumor cells settle in a new area is called metastasis. However, this acquired capability of cancer cells in invasion and metastasis still has not been fully understood, yet. Cancer cells change their shape, interactions, and binding with other neighbor cells by altering their ECM proteins. Also, the proteins affected by this change are cell-cell adhesion molecules (CAMs) such as integrins, cadherins, immunoglobulins, and e-cadherins. The other parameter that cancer cells involve for invasion and metastasis capability is altering their extracellular proteases [7].

Normally, the cells and tissues continuously monitor their environment with the alert of the immune system. The immune system is being responsible for distinguishing and disposing or reducing the possible incipient cancer cells and thus tumor masses that may occur.

However, due to the ability acquired by tumor cells, they somehow escape the detection of tumor cells by different members of the immune system or avoid being killed which leads to limit the immunological killing system, thus escaping eradication [6,7].

Chronic and often uncontrolled cell division of cancer cells which is the main and important acquired ability of the cancer cells in neoplastic disease is based not only on the deregulation of cell division but also on certain cell adjustments in energy metabolism and thus fueling cell division and growth. Normal cells transforms glucose molecule to pyruvate along with glycolysis pathway under aerobic environment conditions in the cytosol and then produce ATP with oxidative phosphorylation in mitochondria. However, under aerobic conditions, cancer cells make glycolysis more advantageous and limit oxidative phosphorylation (OXPHOS) energy metabolism which is more efficient energy pathway to produce ATP. Otto Warburg was first discovered the ability of cancer cells to change their glucose-dependent metabolism by the means of energy production which is called “aerobic glycolysis” [7].

Genomic instability of cancer cells resulting from mutations occurring in neoplastic cells is considered another hallmark of cancer. These cancer cells, which have a mutant genotype, become more advantageous than other cells, thereby ensuring their dominance in the local environment with more proliferation. Changes in tumor suppressor genes also result from many inheritable epigenetic factors such as histone modification and DNA methylation. The presence of "caretaker" genes of great importance in the cell, such as TP53, can detect DNA damage, activate DNA repairing mechanisms, directly repair the damaged DNA, and inactivate molecules that may induce mutation before DNA is damaged. Furthermore, these “caretaker” genes also play a role as tumor suppressors in this mechanism, and their function may be lost during tumor progression, such losses may be caused by either inactivating mutations or epigenetic factors [7,8].

The last hallmark of cancer cells is pointed out as tumor-promoting inflammation. The formation of inflammation provides bioactive molecules to the environment, covering the environment where the tumor is located which includes growth factors that ensure the continuation of the proliferative signal pathway, survival factors limiting cell death, ECM modifying enzymes contributing to angiogenesis, proangiogenic factors, metastasis and invasion factors, and epithelial-mesenchymal transition (EMT) activating signals and many more pathways [9,10]. Apart from these, the ROS produced by the inflamed cells creates a

mutagenic effect on the cells at close range and genetically accelerates the formation of malignancy of tumors [7].

1.1.2. Cancer Metabolism

Normal eukaryotic cells, in the existence of oxygen, after performing the glycolysis step in the cytosol, the pyruvate from the glycolysis enters the Krebs cycle, resulting in the formation of NADH, thereby generating mitochondrial oxidative phosphorylation to maximize the ATP production while producing a minimal amount of lactate [11]. Unlike normal cells, cancer cells perform lactic acid fermentation by increasing glucose uptake, rather than the oxidative phosphorylation step, which should normally proceed, even if oxygen is present in the environment. This phenomenon, also called aerobic glycolysis, shown by most cancer cell types such as breast, lung, colorectal, and glioblastoma, is called the Warburg effect. This anomalous metabolism demonstrated by cancer cells has been presented as a hypothesis by a German scientist Otto Warburg more than 80 years ago, and subsequent studies have confirmed its accuracy [12]. Otto Warburg marked the time with his striking discovery of the metabolism of cancer cells in the 1920s. Through this important study, he became the pioneer of the subject of cancer respiration, which is still under investigation [13]. Normal cells are stimulated by growth factor signals from the environment and then take up nutrients to perform cell division. There is a control mechanism to prevent uncontrolled cell division, even if the constant supply of nutrients in the environment exceeds the nutrient limit required for basic cell division.

Unlike normal cells, cancer cells divide rapidly by overcoming this controlled cell division mechanism, which is dependent on growth factor signals. As a result of genetic mutations that destroy functions of receptor initiated signaling pathways, cancer cells have an uncontrolled cell division mechanism [11]. Warburg has suggested that cancer cells which have dysfunctional mitochondria, prefer aerobic glycolysis instead of complete mitochondrial oxidation of glucose. Following these important findings by Warburg, many working groups, especially the studies conducted in the last 10 years, aimed to better understand the Warburg Effect and to find out the causes of cancer glucose metabolism. However, recent studies have shown that many types of cancer cells that exhibit the Warburg Effect may not have impaired mitochondrial functions. Normal cells perform mitochondrial

oxidative phosphorylation to obtain 36 ATPs per glucose. However, cancer cells with Warburg Effect prefer aerobic glycolysis to obtain only 2 ATPs per glucose (Figure 1.3). Although the amount of ATP obtained per glucose is not efficient, the rate of metabolism in glycolysis dependent cancer cells is suggested as 10-100 times faster than the oxidative phosphorylation in mitochondria [14].

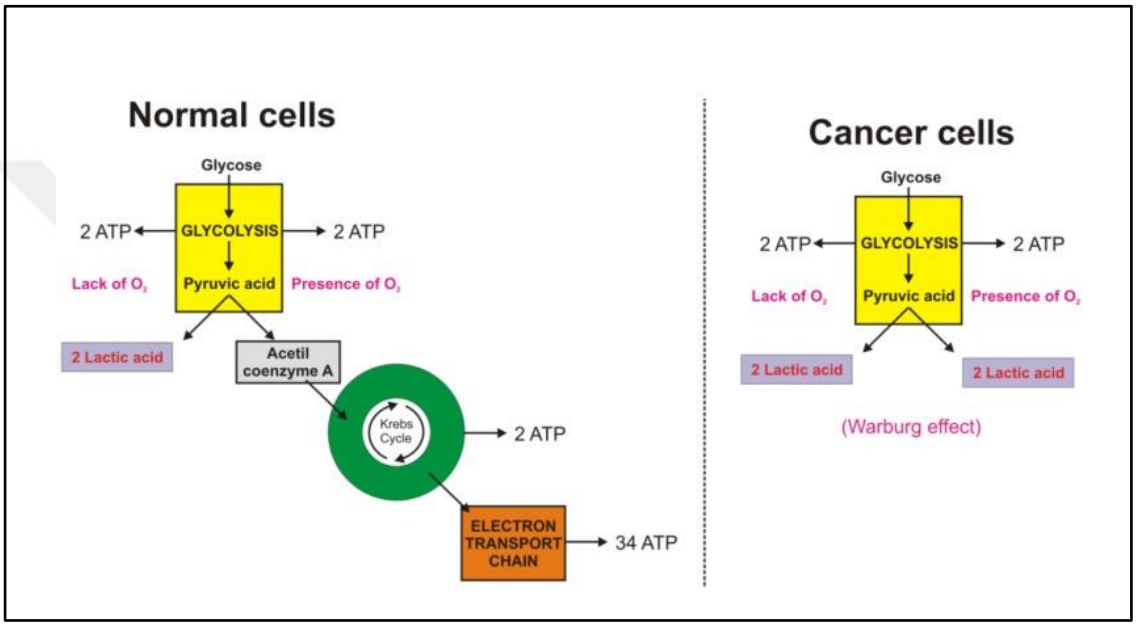

Figure 1.3. Summary of difference in energy production mechanism between normal and cancer cells [15].

Why cancer cells choose a less efficient metabolism has become a popular research question. According to the findings, a fast but low yielded ATP production creates a significant advantage in the competition for energy stocks, which are shared and limited. Cancer cells also compete with their environment as they have a limited presence of glucose in a microenvironment in which normal cells and immune components are present. In another hypothesis, the Warburg Effect is proposed to be a mechanism by which cancer cells adapt to meet the cellular needs of their uncontrolled and rapid cell proliferation. The use of this excess carbon by cancer cells is used for biomass production to maintain cell proliferation. Therefore, proliferating cells produce large amounts of nucleotides, amino acids, lipids, and proteins. The anabolic pathways, for instance, pentose phosphate pathway (PPP) for

nucleotide and aminoacid synthesis, glutamine uptake for providing lipid synthesis, and ATP-citrate lyase (ACL) for the synthesis of acetyl-CoA from citrate to produce lipids is crucial for in the cancer metabolism and essential for rapid tumor growth [16]. Both glucose and glutamine uptake appears to be critical for the production of lactate which the conversion of lactate involves lactate dehydrogenase (LDH). This enzyme serves as an important key point to impair cell proliferation of cancer cells by inhibiting LDH [17].

As a result of the abnormal metabolic activity of cancer cells and lactate produced by high-rate glycolytic flux, the pH in the tumor microenvironment decreases, and this is called acidification of the microenvironment. Based on a hypothesis, cancer cells benefit from the acidic environment in a way that H^{+1} ions alter the tumor microenvironment and tumor stroma in order to increase their invasiveness [14]. Recent studies suggest that hypoxia-inducible factor 1 alpha (HIF1α) and also Myc oncogene which are known as master transcription factors are overexpressed in cancer cells, and they take part in the Warburg effect mechanism. HIF1α and its relationship with cancer metabolism have been studied in recent years. Its activity in cancer metabolism is generally related to the upregulation of plasma membrane glucose transporter 1 (GLUT1), many enzymes of glycolysis, and all pyruvate dehydrogenase kinases (PDKs). Hereby, the inhibiting pyruvate dehydrogenase complex (PDC), which generally generates acetyl-CoA by pyruvate, OXPHOS is also inhibited and aerobic glycolysis rate is increased resulting in acidosis [18].

It is very important to maintain reactive oxygen species (ROS) at a certain level. Excessive amounts of ROS can damage the cell membrane, as well as damage the important building blocks of the cell, such as nucleic acids. Insufficient ROS levels inhibit cell proliferation by affecting important signaling pathways for cell growth. In cancer cells, the ROS level is maintained at an elevated but controlled level for cell growth and division. In this way, the cells not only avoid oxidative damage caused by ROS but also increase metastasis and invasion. The alteration of ROS production due to mitochondrial redox potential changes that are caused by the Warburg effect provides another survival mechanism for cancer cells [19].

1.2. COLORECTAL CANCER

As reported by the organization of Global Cancer Incidence, Mortality and Prevalence (GLOBOCAN) statistical data, colorectal cancer (CRC) ranks the second cancer type among women with 614.000/year cases in worldwide while the third in men [20]. According to studies, the rate of incidence of CRC is higher in modern nationalities for instance Finland, Canada, Sweden, and some parts of Europe, on the other hand, less developed countries have reduced survival rates. For the last decade, decreasing risk factors such as cigarette smoking or consumption of red meat, as well as the development and improvements of new screening tests, have reduced the incidence and mortality rates of colorectal cancer worldwide [21]. On the contrary, there is a rapid increase observed in the incidence and mortality rate of CRC in less developed countries particularly in Asia, South Europe, and America. When statistical data profiles are evaluated, CRC is predicted to reach 2.2 million new cases and 1.1 million cancer-related death by 2030, with a sharp rise of 60 percent [22]. According to the Turkey statistics agency data (TÜİK), colorectal cancer is the most common cancer type in both males and females in 2015 [4].

1.2.1. Pathogenesis of Colorectal Cancer

The large intestine, colon, is an abdominal organ that is approximately 100-150 cm in an adult body. The colon begins with the caecum (blind-ended pouch) part and continues with the ascending, transverse, descending, and sigmoid parts that cover the rectum and anal canal parts (Figure 1.4) [23,24]. The anatomic terminology of the large intestine was given corresponding to their location and configuration in the organ. All of the organs are covered by the endoderm and autonomic nerves. The mucose of the colon consists of two layers: one of the layers is circular and the other one is the longitudinal smooth muscle cells. The blood supply is provided to the colon by branches of the superior mesenteric artery. This part of the colon contains caecum to splenic flexure. The other branch that supports blood supply is the inferior mesenteric artery which contains three parts: the descending colon, the sigmoid area, and the rectum. The middle and inferior rectal arteries are taken part in supplying blood to the lower part of the rectum. This vascularization of the colon arises different types of malignancies in CRC. These are the cancer of the left colon which presents in the descending

and sigmoid region in the colon, cancer of the right colon that presents in the caecum to the splenic flexure, and cancer of the rectum that presents within the eight cm of the anal verge. Most of the venous blood that passes through the organ by the portal system leads to blood to reach the liver that is considered to be the main metastasis area of colorectal cancer [25].

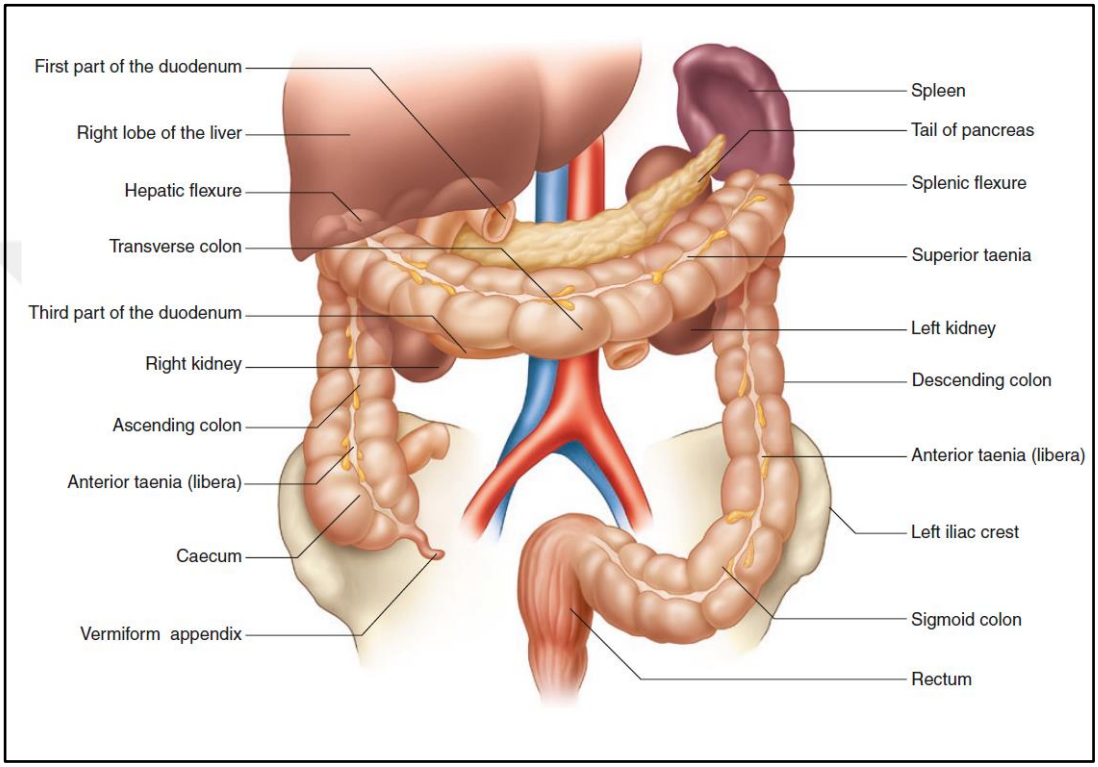

Figure 1.4. Anatomy of the colon [23].

Although CRC is a very common type of cancer, it is also considered one of the most preventable types of cancer because it also has a slow polyp-cancer progression. Deaths related to colorectal cancer annually are calculated to be approximately more than 8 percent. Besides, the risk of both women and men getting colorectal cancer throughout their lifetime is approximately 4.3 percent [26]. CRC often begins as benign neoplasms, resulting in the conversion of serrated polyps and tubular adenomas to CRC after many years. Since the disease has a long process of progression, it allows the detection and removal of polyps in patients before polyps transform into malignant tumors [27]. According to recent studies, genetic factors and also environmental factors are employed in the conversion of polyps to CRC. This type of cancer progression can be divided into two types: sporadic colorectal

cancer that is not due to the inherited gene mutations and inherited colorectal cancer [28]. In particular, life-style and dietary risks such as unbalanced energy intake, unsaturated fat-containing foods, red meat consumption, low physical activity, and excessive alcohol consumption have significant effects on sporadic colorectal cancer disease progression. A recent review suggests that intestinal flora and local inflammation are also crucial in the development of CRC. Even though the causes of colorectal cancer are not known precisely, it has been said to have many risk factors such as age, diet, familial, and individual colorectal cancer history. Besides, approximately 2-5 percent of all colorectal cancer cases were identified to be caused by inherited syndromes [26,29].

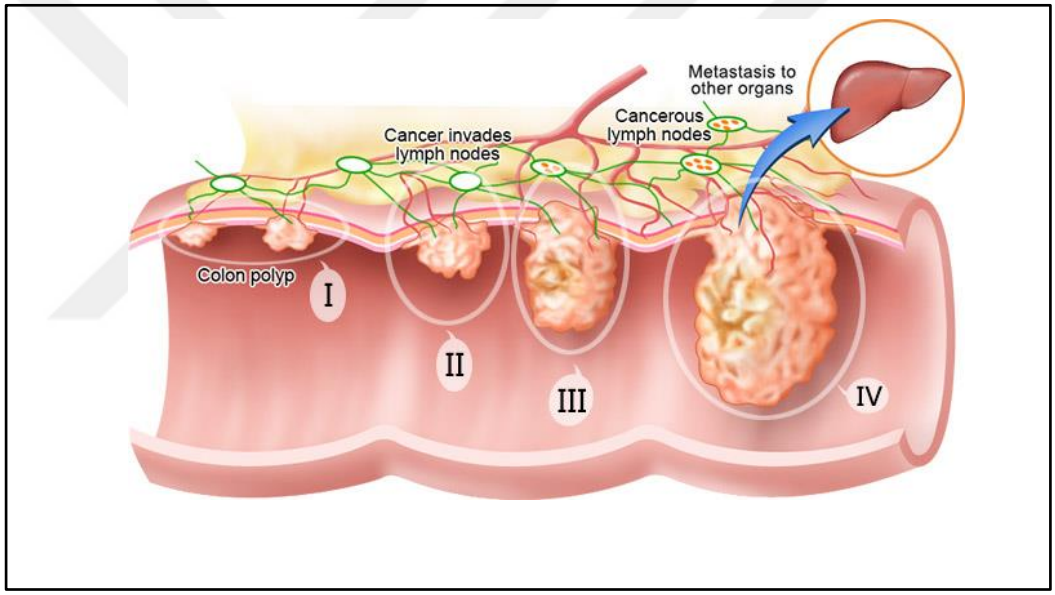

Figure 1.5. Stages of colorectal cancer [32].

In addition to environmental factors, inherited cancer syndromes include Peutz-Jegehers syndrome (PJS), familial adenomatous polyposis (FAP), juvenile polyposis syndrome (JPS), hereditary CRC, and Cowden disease. Molecular abnormalities such as DNA abnormalities (aneuploidy, microsatellite imbalance), epigenetic changes (chromosomal instability (CIN), and CpG Island Methylator Phenotype, (CIMP)) serve importance on the heterogeneity of polyp-CRC transformation [27,30]. It has been estimated that 5-10 percent of cases of colorectal cancer have been associated with inherited genetic defects, but most of them are sporadic which is the most common type of disease that includes both environmental factors

and genetic defects which arises from environmental exposures. Almost 70 percent of colorectal cancer cases are suggested to depend on lifestyle and, most importantly, diet [31]. The spread of CRC usually occurs in the form of local invasion to contiguous organs or the form of lymphatic or hematogenous invasion. Three different classification systems are used in the staging of CRC cases. These systems include Dukes, Astle-Coller, and TNM system. Since the TNM classification system, which was created by the American Joint Committee on Cancer (AJCC) and the International Association of Cancer (UICC). This classification system is a more detailed and easier system than other classification systems, the treatment decisions in recent times are determined by using the TNM system. The TNM classification of CRC is based on the presence of primary tumor (T), local lymph node metastases (N), and distant metastasis (M) (Table 1.1 and 1.2) [28,33]. Colorectal cancer is not very likely to undergo metastasis. However, gross metastases usually occur in regional lymph nodes, bones, ovary, and especially in the liver. Other areas of metastasis can be lungs, pelvis, peritoneum, and adrenals in the body [28].

Table 1.1. Definitions of the TNM classification system in colorectal cancer [34].

Primary Tumor (T)	
TX	No assessment of primary tumor
T0	Absence of primary tumor
Tis	*In situ* carcinoma
T1	Tumor spread to the submucosa
T2	Tumor spread to muscularis propria
T3	Tumor spread across muscularis propria and pericolorectal tissues
T4a	Tumor diffused the visceral peritoneum
T4b	Tumor spread or is adherent to different parts, organs, structures directly
Regional Lymph Nodes (N)	
NX	No assessment of local lymph nodes
N0	No assessment of local lymph node metastasis
N1	Presence of one to three local lymph nodes metastasis
N2	Presence of four or more local lymph nodes metastasis
N2a	Presence of four to six local lymph nodes metastasis
N2b	Presence of seven or more local lymph nodes metastasis
Distant Metastasis (M)	
M0	Absence of far-off metastasis
M1	Presence of far-off metastasis
M1a	Presence of metastasis in one organ/area (liver, lung, ovary, nonlocal node)
M1b	Presence of metastasis in more than one organ/area

Table 1.2. Anatomic stages according to TNM classification system [34].

Stage of cancer	**T**	**N**	**M**
0	TX	N0	M0
I	T1-T2	N0	M0
IIA	T3	N0	M0
IIB	T4a-T4b	N0	M0
IIIA	T1-T2 T1	N1 N2a	M0 M0
IIIB	T3-T4a T2-T3 T1-T2	N1 N2a N2b	M0 M0 M0
IIIC	T3-T4	N1-N2	M0
IV	Any of T	Any of N	M1

1.2.2. Screening

It takes years before colorectal cancer starts from early adenoma and turns into invasive cancer. Detecting and eliminating cancer precursors serve importance in reducing the mortality and incidence of this cancer type. Therefore, early diagnosis is very important in colorectal cancer, so patients face a less invasive treatment, thereby reducing the treatment cost with mortality [35]. There are three main types of screening methods currently used for colorectal cancer: non-invasive stool tests, endoscopic examinations, and radiologic examinations. Stool tests include the presence of abnormal DNA, the presence of occult blood (FOBT), immunochemical FOBT (iFOBT), and guaiac-based FOBT (gFOBT). Endoscopic examinations include flexible sigmoidoscopy (FS) colonoscopy. Radiologic examinations computed tomographic colonography (CT) and double-contrast barium enema (DCBE) [36].

1.3. THERAPEUTIC APPROACHES IN COLORECTAL CANCER

It is known that most cancer types have a glycolysis-dependent metabolism called the Warburg effect as revealed in many studies. New and feasible therapeutic strategies to target glycolysis for cancer therapy have been extensively researched in recent studies. Inhibition of certain enzymes in the glycolysis pathway or reducing the presence of glucose in the tumor environment and thereby reducing glucose uptake are considered as promising

approaches [37]. It is unraveled that the efficacy of anti-glycolytic agents such as 2-deoxyglucose (2-DG), rapamycin, dichloroacetate (DCA), lonidamine (LN), metmorphin, and 3-bromopyruvic acid (3BP) against CRC *in vitro* and *in vivo* studies [38,39]. 2-DG that is known as a glucose analog, accumulate by the HKII enzyme, leading to inhibition of the HKII enzyme, thereby causing the death of cells by going to apoptosis. This molecule, which has limited anti-cancer properties but has little toxicity to normal cells, was found to enhance the efficacy of commonly used anti-cancer molecules by using combinations such as cisplatin. In a previous Phase I/II clinical study which includes 100 selected patients, an increase in survival rate was observed when 20 min before irradiation, where 200 to 300 mg 2-DG kg/body weight ratio was given to patients orally [40].

The use of nutraceuticals in cancer prevention and treatment has an important role in cancer metabolism studies since they show fewer adverse effects. The welfare of dietary botanicals, namely plant-derived products in human life, is based on a very long history [41]. Numerous antioxidants have been studied in pre-clinical and clinical studies such as berberine, piperine, curcumin, pomegranate, silymarin, garlic, flavonoids, simvastatin, resveratrol against colorectal cancer. National Cancer Institute and American Cancer Society recommend that increasing natural sustenances uptake reduces the risk of individuals in cancer development. However, in order to include these consumed natural foods as daily supplements or to be included in clinical studies, which components play a role in cancer prevention and their working mechanisms should be determined. According to recent studies, in the last decade, almost 50 percent of the drugs that hold approval of Food and Drug Administration (FDA) consist of natural products, derivatives of natural products, compounds which are based on natural products, or compounds imitating these natural products [31]. It has been found in clinical studies that an alkaloid piperine, the bio-active ingredient of black pepper, has increased the absorption of therapeutic drugs and phytochemical molecules by inhibiting enzymatic drug transforming enzyme reactions in the liver and intestines. Piperine has been suggested to effectively reduce the ROS levels, which is positively affected by the antioxidant status of the cells, in studies conducted for *in vitro* and *in vivo*. thereby protecting against oxidative damage [31]. In many pre-clinical and clinical studies, resveratrol is beneficial in preventing colorectal cancer and many other types of cancer by inducing apoptosis, inflammation, and proliferation. Resveratrol (trans-3,5,4′-trihydroxy-stilbene) is a kind of polyphenolic phytochemicals and is found mainly in skins of grapes and other

nutrients such as peanut, berries, and red wine [42]. In pilot studies in healthy people, the safe and well-tolerated dose of resveratrol was reported as 5 g/day. In a study on 20 colorectal cancer patients, resveratrol was taken 0.5 g or 1 g per day by oral intake 8 days before surgery. There was a 5 percent reduction in tumor sizes examined after surgery [43,44].

1.3.1. 3-Bromopyruvate (3BP)

3BP is a pyruvate mimetic which has high reactive electrophilic alkylation property (Figure 1.6). In the early studies, this small molecule has been used in analytical chemistry studies for affinity labeling of target proteins by taking advantage of the affinity property of 3BP to thiol/cysteine groups in the proteins. Recent studies have revealed that 3BP has shown a severe anti-cancer effect on many cancer types, such as colorectal cancer [45], HCC [46], breast [47], glioblastoma [48]. 3BP causes inhibition of the glycolytic enzyme hexokinase II (HKII), which plays a vital role in the glycolysis pathway, causing ATP depletion in cells and triggering cell death. It also causes dissociation of HKII from mitochondria which plays an important role in mitochondrial membrane integrity (Figure 1.7) [49]. The upregulation of HKII basically in cancer cells leads to increased glycolysis rate and association of HKII with a voltage-dependent anion channel (VDAC) which forms a complex on the cytosolic side of mitochondrial membrane taking part in inhibition of apoptosis pathway by blocking of cytochrome c release from mitochondria [50]. The inhibition of the overexpressed HKII enzyme in solid tumors, disruption of cell energy balance and thus targeting cancer cell death, has become a popular strategy in recent *in vitro* and *in vivo* studies. However, the molecular targets of 3BP are not limited to HKII, but include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mitochondrial succinate dehydrogenase, endoplasmic reticulum (ER), and lysosomes. Since 3BP has a large and important molecular target group, it can efficiently kill cancer cells that need plenty of ATP [51]. 3BP simply affects glucose metabolism, causing ATP production to stop, thereby targeting the Warburg Effect (Figure 1.7). As shown in studies, cancer cells showing Warburg Effect and thus high lactate production, they overexpressed mono-carboxylate transporters (MCTs) to remove excessive lactate amount produced and to avoid cytoplasmic acidification. Because of the MCT overexpression, the 3BP molecule can be successfully taken into the cell through MCTs and show its anti-cancer feature. The reason why 3BP can enter the cell so easily is that only the

Bromine (Br) atom is different from lactic acid and MCTs cannot distinguish the difference. However, MCT-mediated transport does not occur specifically to the 3BP molecule [52].

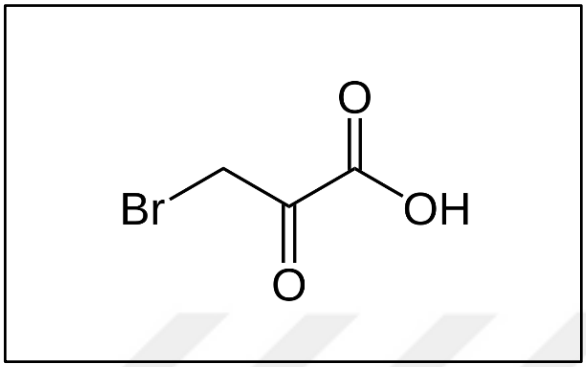

Figure 1.6. Structure of 3BP molecule [50].

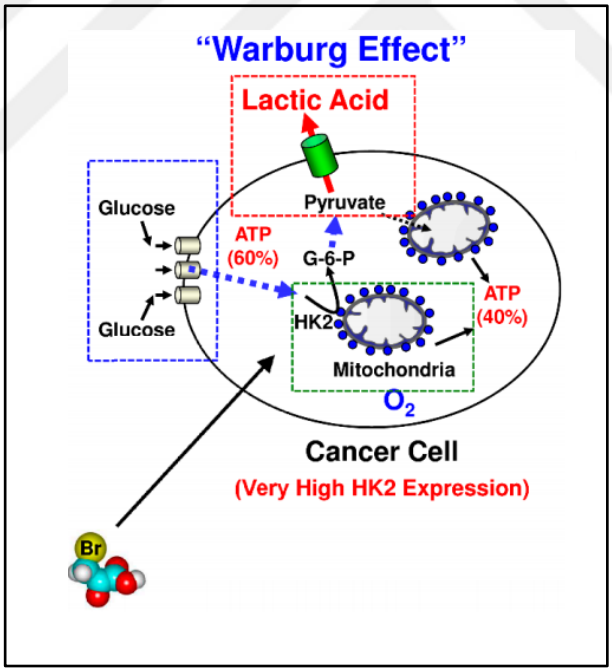

Figure 1.7. The simple mechanism of 3BP on cancer cells [52].

After the many years of research on 3BP high efficacy on multiple cancer types and *in vivo* modeling studies, it has shown as a promising and potent candidate molecule for human cancer patients for clinical trials. There are not any approved clinical trials for 3BP however there are 2 reports on volunteer patients about this small molecule. In 2012, a 16-year-old

patient with HCC was treated with 3BP and the patient was survived 2 more years than expected with no adverse effects and increased life quality. In 2014, 28-year-old patient with metastatic melanoma in stage IV was treated by using paracetamol to decrease glutathione (GSH) levels in blood and 3BP to decrease LDH levels in serum. This combination of paracetamol and 3BP lead to a sharp decrease in LDH levels in serum [53].

1.3.2. Curcumin

Curcumin, or commonly known as turmeric, is a commonly used spice obtained from the rhizomes of a plant called *Curcuma longa*, which belongs to a ginger family (Zingiberaceae) [54]. The active ingredient of turmeric from *Curcuma longa*, has a distinctive vivid yellow-orange color originating from oil-soluble natural polyphenolic curcuminoids. Other curcuminoids found in turmeric except for curcumin are demethoxycurcumin (DMC), and bisdemethoxycurcumin (BDMC) [55]. The story of the use of curcumin in various fields dates back to 4000 years. It is used both in traditional Indian cuisine and Ayurveda medicine, as well as in many parts of the Asian region, especially in China, as both spices and colorants. Ayurveda medicine is used as an ancient medical system in India and is based on the healing properties of medicinal plants such as turmeric in patients. In this traditional medical approach, turmeric has been used to treat many diseases such as digestive system disorders, insect bites, wound healing, chickenpox, urinary tract, and eye infections [56]. Despite the long history of curcumin, it took up to the 1800s for scientists to isolate curcumin. Then, in 1870, the crystal form of curcumin was obtained, and in 1910, the unique physicochemical and biological structure of curcumin was illuminated. Curcumin, 1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene3,5 dione, ($C_{21}H_{20}O_6$) is a hydrophobic polyphenol which has a molecular weight of 368.37 g/mol^{-1} and a melting point of 183°C [10]. It has 2 phenolic rings in its chemical structure as shown in Figure 1.8 [54,58].

Curcumin is not stable in basic pH, but stable in neutral and acidic environments. Its presence in the basic environment leads to degradation of curcumin and formation of end products such as ferulic acid, feruloyl methane, and vanillin. The availability of fetal bovine serum (FBS), or antioxidant molecules for instance ascorbic acid and N-acetylcysteine in the environment, prevents curcumin from becoming degraded [58]. Curcumin exhibits a large number of biological activities and has a pharmacologically safe profile, which has attracted

more attention and research in the last 30 years [59]. In recent studies, curcumin, which is well-known for its strong antioxidant, anti-cancer, and anti-inflammatory features, has also been shown to have anti-bacterial, and anti-viral effects. Since curcumin is present in large quantities in nature and also, it has low cytotoxicity and negligible side effects. Besides, it plays a significant role in the prevention and treatment of various diseases such as autoimmune, neurodegenerative, inflammatory, skin, malignant, and heart diseases [60].

So far, many scientific studies have been presented that curcumin has anti-cancer properties over different pathways. *In vivo* and *in vitro* studies have revealed that curcumin affects many types of cancer, such as colon, liver, skin, breast, kidney, and HCC by stoping angiogenesis, decreasing tumor promotion and initiation, blocking metastasis, decreasing cell proliferation and invasive effects [61,62]. While curcumin can activate the cell survival mechanism by proteasome stimulation according to the dosage it is used, it can also trigger apoptosis by proteasome inhibition. In addition, low doses of curcumin cause oxidative stress and apoptosis, while high doses of curcumin cause decreased ROS production, ATP reduction, and necrotic cell death. While it can provide cell death by mitochondria-dependent and mitochondria-independent mechanisms, it can cause cell death by activation of the mitotic catastrophe which is identified by the formation of multinucleated and giant cells in cells that are apoptosis-resistant [63].

In addition to the *in vitro* and *in vivo* studies about curcumin, several clinical trials have been conducted in order to detect its safety and efficacy. In the previous study related to dose escalation on 24 human subjects, a well-tolerated high dose of curcumin was found as of 12 g per day [64]. In a previous phase I clinical trial study, 126 selected CRC patients were given of curcumin at a concentration of 360 mg single dose three times per day for 10-30 days before surgery. As a result, the overall health of cancer patients improved, serum TNF-α levels decreased, apoptotic cell count increased, p53 expression increased [65]. In phase II clinical trial, selected colorectal cancer patients were administered with oral administration of curcumin for 30 days at 2 g/day or 4 g/day. As a result, aberrant crypt foci (ACF) numbers decreased in patients who received 4 g/day [66].

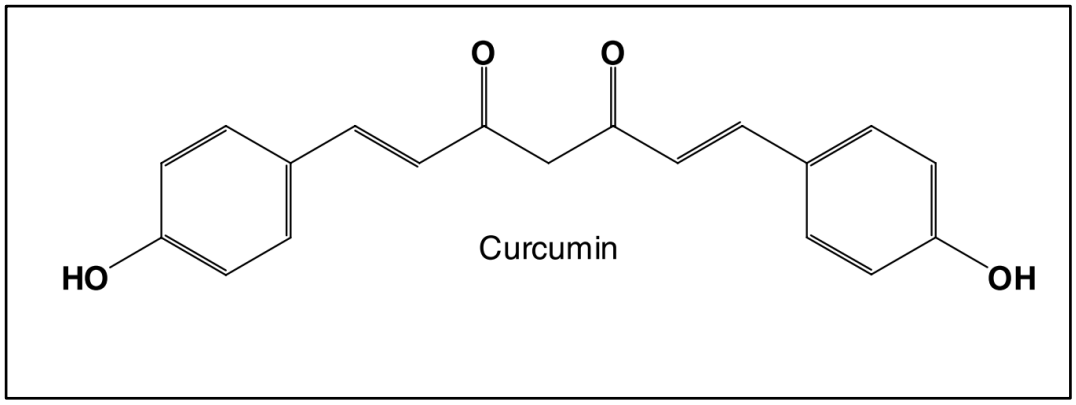

Figure 1.8. Molecular structure of curcumin [55].

1.4. MITOCHONDRIAL DYSFUNCTION IN CANCER

Mitochondria were defined by the name 'sarcosomes' during the studies in human muscle tissue by Rudolf von Koelliker, a Swedish anatomy specialist, in 1857. Later, in 1898, a scientist named Carl Brenda called the word “mitochondria” [67]. Mitochondria are important organelles in a cell that are known for their traditional role of “powerhouses” of the cell. These ancient organelles have the double membrane; inner membrane and outer membrane with an intermembrane space between them (Figure 1.9). They are responsible for producing ATP by OXHPOS and many other vital cellular processes such as controlling ROS detoxification, cell death mechanisms, hormonal signaling, amino acid, and fatty acid metabolism [67,68]. The outer mitochondrial membrane (OMM) located between the mitochondria and cytosol and provides the passaging of necessary metabolites from the membrane by VDACs and translocases. Mammalian mitochondria have their DNA (mtDNA) which is enclosed by IMM in the mitochondrial matrix. The circular double-stranded mtDNA is approximately 16.6 kb. The inner mitochondrial membrane (IMM) contains numerous cristae structures by folding of IMM in order to increase surface area for OXPHOS complexes for the generation of ATP [69].

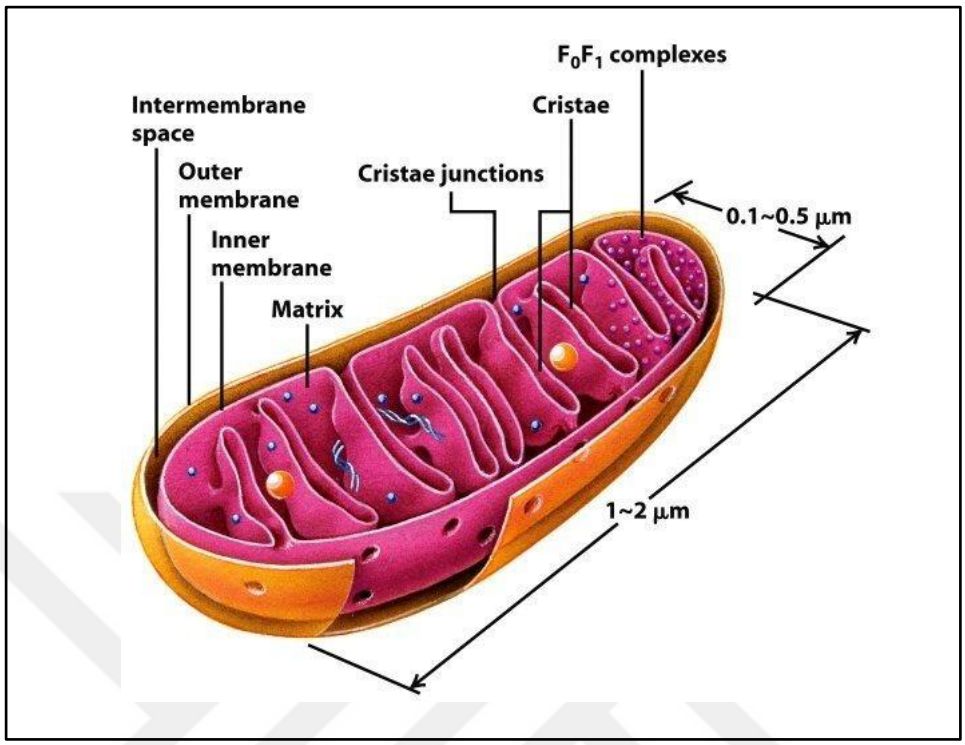

Figure 1.9. Structure of mitochondrion [70].

The number of structural OXPHOS subunit genes identified to date is known as 92 in total. While 13 of these known subunits are encoded by mtDNA, 79 of them are encoded by nuclear DNA (nDNA). Complex I (NADH: ubiquinone oxidoreductase or NADH dehydrogenase) is known as the largest complex of the OXPHOS system, it has 44 subunits consisting of 14 enzymatic core units (7 from nDNA and 7 from mtDNA), and 30 nDNA accessory subunits. Complex II (succinate dehydrogenase or succinate:ubiquinone oxidoreductase) has 4 subunits and is completely encoded by nDNA. Complex III (cytochrome bc1 or ubiquinol:cytochrome *c* oxidoreductase) has 11 subunits: 1 of them is encoded by mtDNA and 10 of them by nDNA. In addition, Complex IV (cytochrome c oxidase) contains 11 nDNA encoded subunits and 3 mtDNA encoded subunits. Finally, Complex V (ATP synthase or F_0F_1-ATP synthase) consists of 19 subunits, 2 of which are encoded by mtDNA and 17 of them are nDNA encoded (Figure 1.10) [71].

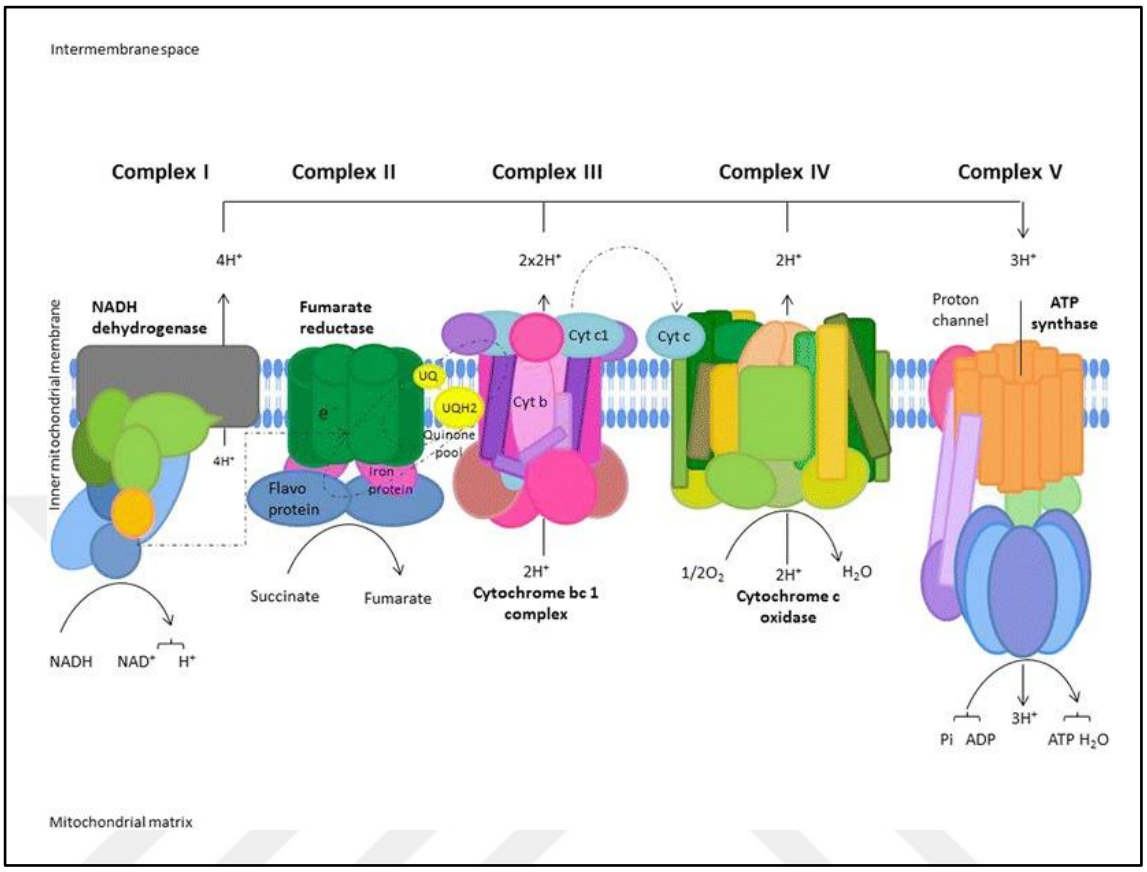

Figure 1.10. Oxidative phosphorylation system (OXPHOS) [72].

The OXPHOS system uses NADH and $FADH_2$ reducing equivalents as electron donors that are produced in the glycolysis pathway or citric acid cycle in order to pump out the electrons from the inner mitochondrial membrane to inner membrane space. This generated a proton electrochemical gradient is mainly utilized for the production of ATP and importing proteins and Ca^{2+} into mitochondria [73]. NADH and $FADH_2$ enter the electron transport chain (ETC) via passing their electrons by firstly to the Complex I and then Complex II. When NADH and $FADH_2$ participate in the ETC, they are oxidized and both pass their electrons to the ubiquinone. Also, Complex II oxidizes succinate from the TCA cycle to fumarate. Complex II does not have a proton pump role in the OXPHOS system thus it does not take part in the proton gradient formation. Then, Complex III pumps two protons to the intermembrane space by oxidizing ubiquinone to ubiquinol structure. The electrons from the ubiquinol are passed through the cytochrome c carrier by cytochrome b and c_1 parts of the Complex III. When cytochrome c in the Complex IV accepts the electrons from Complex III, they are

transferred to the oxygen molecule in order to generate two water molecules. During this process, protons are pumped to the intermembrane space. Then, the electrons are transferred to their final acceptor Complex V for the production of ATP. The energy that is stored in the proton gradient is used to turn ADP to ATP. When protons are crossing the membrane through the proton channels, it starts to rotate to allow the passing of protons into the intermembrane space. While this rotation, a phosphate is attached to the ADP molecule thus ATP molecule is produced [74].

Mitochondrial dysfunction might be generated by the mutations in mtDNA and nDNA. It has been determined that these functional disorders in mitochondria is crucial for many mitochondrial related diseases for instance aging, age-related diseases, neurodegenerative diseases, obesity, cancer [75]. In many studies, it was determined that defects such as mutations, insertions, deletions, and copy number decreases in mtDNA are seen in many cancer types such as prostate, HCC, breast, and colorectal. The mtDNA mutations occurring in Complex I, III, IV, and V subunits, are important especially in proton gradient, are suggested to be effective in tumorigenicity and their aggressiveness [76]. For example, in a comprehensive study to investigate mtDNA mutations in colorectal cancer cell lines, mutations were found in ND1, ND4L, and ND5 subunits of Complex I, and COXI, COXII, and COXIII subunits of Complex IV [77]. In particular, the reduced function of some subunits of Complex I and Complex IV caused by the mtDNA mutations have caused a tumor-promoting effect by causing an excess of cytosolic and mitochondrial ROS as a result of electrons escaping from the respiratory chain [78].

Another reason for mitochondrial dysfunction is derived from the excessive generation of reactive species (RS) microenvironment of tumor mass. Oxidative stress arises from the imbalance between RS and antioxidant defense systems, resulting in increased cellular RS. While superoxide anion, peroxide, hydrogen peroxide, singlet oxygen, and hydroxyl radicals are types of ROS, nitric oxide, reactive halogens, and sulfur species are reactive nitrogen species (RNS). The antioxidant defense system in cells consisting of enzymatic and non-enzymatic mechanisms, and they act as mitochondrial, cellular membrane, and extracellular. The most important mitochondrial antioxidant enzymes are manganese superoxide dismutase (mnSOD2 or SOD2) and glutathione peroxidase (GPx) [79]. The SOD2 enzyme is one of the most effective enzymes in the antioxidant mechanism, and it has been found that over-expression of this enzyme causes retarded tumor growth on several cancer types.

Oxidative stress damages the structures in the cells and contributes to the formation of cancerous structures by causing somatic mutations. One of the reasons for the elevated ROS level of cancer cells may be the hypoxic environment in which they are located. Increasing ROS with hypoxic microenvironment contributes to increased ROS levels by causing alterations in mtDNA thus establishing a vicious cycle of ROS production and oxidative stress in cancer cells [79,80].

1.5. AIM OF THIS STUDY

Our study aims examining the combinatorial effect of 3-bromopyruvate (3BP) and curcumin (CUR) for cellular, mitochondrial, and glycolytic metabolism of HCT116, colorectal cancer cell line. In order to reveal the anti-proliferative effect of 3BP and CUR cell proliferation is investigated along with microscopical observations of cell morphology. According to the data obtained from cell proliferation assay and microscopic observations, flow cytometric analysis is performed to examine the mechanism of death. Intracellular ROS levels are examined to investigate stress factors that may cause cell death. Later, mitochondrial mass content, physiological changes and its function upon COT is studied by using confocal imaging. Also, alterations in mitochondrial bioenergetics are examined through studying protein expressions of oxidative phosphorylation complexes. Finally, glycolytic energy production is assessed through lactate dehydrogenase-A (LDH-A) protein expression levels upon treatments to investigate Warburg effect.

2. MATERIALS AND METHODS

2.1. CELL CULTURE

Human colorectal cancer cell line, HCT116 (ATCC, USA) were grown in high glucose Dulbecco's Modified Medium (DMEM) (Gibco, USA) plus 10 percent fetal bovine serum (FBS) (Gibco, USA), 1 percent 10.000 units/mL penicillin and 10.000 µg/mL streptomycin (Gibco, USA) at 37°C and 5 percent CO_2 (Nuaire NU5510/E/G, USA) [81].

2.2. PREPARATION OF TREATMENTS

3-bromopyruvic acid (3BP) was dissolved in incomplete low glucose DMEM (Gibco, USA) at 50 mM concentration and filtered by using 0.22 µm filter before aliquot. 3BP aliquots were kept at -20°C for further experiments. Curcumin (CUR) was dissolved in DMSO at 50 mM concentration and protected from light exposure. Each treatment was freshly diluted to required concentrations by using complete low glucose DMEM before each experiment.

HCT116 colorectal cancer cells were treated by using 50 µM 3BP, 50 µM CUR, and their combination in low glucose DMEM for 24h at 37°C and 5 percent CO_2 for experiments.

2.3. CELL PROLIFERATION ASSAY

Cell proliferation of HCT116 cells after treatment with 3-bromopyruvic acid (3BP) (Sigma Aldrich, USA) and curcumin (CUR) (Sigma Aldrich C1386-50G, USA) was determined by using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, USA) cell viability assay. Basically, MTS tetrazolium is converted in to a colored soluble formazan product which gives an absorbance in tissue culture medium. Briefly, the density of 10.000 cells/well was seeded in a 96 well-plate and cells were allowed to adhere for 24h at 37°C and 5 percent CO_2. After 24h, cells were treated with various concentrations of 3BP and CUR to determine the combined concentration of 3BP and CUR. After incubation, the medium was discarded and MTS solution (10 percent)

was added into wells (2h dark, 37°C incubation). The absorbance of culture media was measured at 590 nm via BioTek ELx800, USA. The absorbance of the untreated control group was taken as 100 percent [82,83]. The cell viability of cells after treatments were calculated by using the equation given below:

$$\text{Cell viability\%} = \frac{(\text{Absorbance of treatment - Absorbance of background})}{(\text{Absorbance of control - Absorbance of background})} \times 100 \qquad (2.1)$$

2.4. ANNEXIN V-PI STAINING

Treatmen- induced mechanism of cell death with 3BP, CUR, and COT in HCT116 cells were analyzed by using Annexin V-FITC Apoptosis Staining / Detection Kit (Abcam, ab14085, USA) according to manufacturer's protocol [84]. Basically, Annexin V binds to phosphatidylserine (PS) molecules, which are located on the inner side of the cell membrane. PS translocates from inner to outside of the cell membrane in the early stages of apoptosis. On the other hand, PI dye only enters damaged cells that lost their membrane integrity. Briefly, HCT116 cells ($2x10^5$/well) were seeded into 12-well plate and incubated overnight for attachment. Cells were treated with 50 µM 3BP, 50 µM CUR, and their combinations for 24h. After treatment, cells were harvested and resuspended in 300 µl of 1X Binding Buffer. Then, cells were dyed by using 3 µl of Annexin V-FITC and same amount of propidium iodide (PI) at room temperature for 5 min in dark. Cell analysis was done (Ex = 488 nm, Em = 530 nm) by Guava easyCyte 5 flow cytometry (Merck, Germany). The obtained data were analyzed by using Guava InCyte (Merck, Germany) software. The data was obtained and evaluated by using Guava InCyte (Merck, Germany) software.

2.5. INTRACELLULAR ROS MEASUREMENT

Intracellular ROS levels of HCT116 cells were detected by using Intracellular ROS Detection Kit (Abcam, USA) according to manufacturer's protocol [85]. Basically, the fluorogenic dye 2',7'-dichlorofluorescein diacetate (DCFDA) is deacetylated by esterases which is followed by oxidation ROS molecule and then converted into a fluorescent and detectable molecule, 2',7'-dichlorofluorescein (DCF). HCT116 cells at a $5x10^5$/well

concentration were seeded in a 6-well plate and incubated overnight for attachment. Cells were treated with 50 μM 3BP, 50 μM CUR, and their combinations. After treatment, cells were trypsinized and harvested cells were dyed with DCFDA (2',7'–dichlorofluorescein diacetate) fluorogenic dye at 37°C for 30 min at dark. As a positive control, tert-butyl hydroperoxide (TBHP) was used. After incubation, intracellular ROS levels after treatments were detected with FACSCalibur flow cytometer (BD Biosciences, USA). The data was obtained and analyzed by using CellQuest Pro software (BD Biosciences, USA).

2.6. PROTEIN QUANTIFICATION

Protein isolation of the cell pellets with slightly modified method of Hur *et al*., (2009) was conducted by using RIPA (radioimmunoprecipitation assay) cell lysis buffer that contains 1 percent (v/v) protease inhibitor (PI) cocktail, 1 percent (v/v) deacetylation inhibitor cocktail (DIC), 1 percent (v/v) PMSF and 1 percent (v/v) sodium orthovanadate (Santa Cruz Biotechnology, USA) [86]. Cell pellets were dissolved in RIPA buffer and incubated on ice for 15 minutes. After incubation, samples were centrifuged (HETTICH Rotina 35, Germany) at 14000xg at 4°C for 15 minutes. The supernatant was transferred to a new centrifuge tube for protein concentration analysis. The protein concentration of samples was measured by using Pierce BCA Protein Assay Kit (ThermoScientific, USA) according to manufacturer's protocol and calculated by using the standard curve of bovine serum albumin (BSA) [87]. After isolation, isolated protein was put at -20°C until later use.

2.7. IMMUNOBLOTTING

Isolated protein samples (25 μg) were separated with slightly modified SDS-PAGE method of Hur *et al*., (2009) by using a 12 percent SDS-polyacrylamide gel for 120 min at 120 V [86]. Polyvinylidene difluoride (PVDF) membrane was used to transfer proteins from the gel by using Pierce G2 Fast Blotter semi-dry transfer system (ThermoScientific, USA). This membrane was blocked by using 5 percent milk powder solution prepared with TBS-T (Tris-buffered saline buffer containing 0.1 percent Tween-20) for 1h under room temperature. The membrane was washed by using TBS-T for 3 times for a total of 45 min. PVDF membranes were incubated 16 h with Lactate Dehydrogenase-A (LDH-A) antibody (Sigma Aldrich,

USA) at 1:1000 dilution and Total OXPHOS Rodent antibody cocktail (Abcam, USA) at 1:5000 dilution. Beta-actin antibody (Cell Signaling, USA) at 1:5000 dilution was used as a loading control for total cell lysate. After corresponding secondary antibody incubation was completed, the membrane was imaged via Clarity Western ECL Substrate (Bio-Rad, USA), visualized with ChemidocTM XRS+system (BioRad, USA), and processed with ImageLab software (Bio-Rad, USA).

2.8. CONFOCAL IMAGING

2.8.1. Mitotracker Green FM Staining

In order to characterize mitochondrial mass in HCT116 cells after treatments, MitoTracker Green FM dye was used which accumulates in the mitochondrial matrix [88]. Briefly, HCT116 cells at a $2.5x10^5$/well concentration were seeded on coverslips which were placed in a 6-well plate and incubated overnight for attachment. Cells were treated with 50 μM 3BP, 50 μM CUR, and their combinations. After treatment, each well was washed with PBS and treated with 100 nM of Mitotracker Green FM (Life Technologies, USA) dye in the incomplete medium at 37°C for 30 min at dark. After, each well was washed with 1X PBS for 5 times with a 5-minute washing interval. Coverslips were placed facing downwards on the cover slides and cells were imaged (ex/em = 490/516 nm) by using Zeiss LSM 800 confocal microscope (Zeiss, Germany) by using ZEN 3.0 Blue Edition (Carl Zeiss Microscopy, Germany).

2.8.2. Rhodamine 123 Staining

In order to characterize mitochondrial inner membrane potential in HCT116 cells after treatments, Rhodamine 123 dye was used which is a fluorescent cation accumulates in the mitochondrial matrix in energized mitochondria [89]. HCT116 cells at a $2.5x10^5$/well concentration were seeded on coverslips which were previously placed in a 6-well plate and cells were incubated overnight for attachment. Then, cells were treated with 50 μM 3BP, 50 μM CUR, and their combinations. After treatment of cells, each well was washed with PBS and treated with 5 μg/ml concentration of Rhodamine 123 (Sigma, USA) dye in the

incomplete medium at 37°C for 30 min at dark. After, each well was washed with 1X PBS for 3 times with a 5-minute washing interval. Coverslips were placed facing downwards on the cover slides and cells were imaged (ex/em = 507/529 nm) by using Zeiss LSM 800 confocal microscope (Zeiss, Germany) by using ZEN 3.0 Blue Edition (Carl Zeiss Microscopy, Germany).

2.9. STATISTICAL ANALYSIS

All experimental data analysis were done by using GraphPad Prism 6 software version 6.01 (GraphPad Software, Inc., San Diego). Statistical differences among groups were investigated by using one-way ANOVA coupled with the post-hoc tests by Tukey and two group statistical differences were calculated by using Student's t-test. All repeated experiments were conducted in triplicate. *P*-value lower than 0.05 was considered as statistically significant.

3. RESULTS

3.1. CELL PROLIFERATION

The anti-proliferative effects of 3BP and CUR on HCT116 cells were determined by using MTS cell proliferation assay. First, in order to determine the combination dose of 3BP and CUR, HCT116 cells were subjected to various concentrations of 3BP and CUR between 12.5-200 μM for 24h. As seen in Figure 3.1, both 3BP and CUR lead to a decrease in cell viability of HCT116 cells dose-dependently. According to the results, 50 μM 3BP with cell viability of 55 percent and 50 μM CUR with cell viability of 51 percent revealed a significant cell proliferation inhibition compared to the nontreated control group. As a result, 50 μM 3BP and 50 μM CUR were selected for further combination (COT) group treatments.

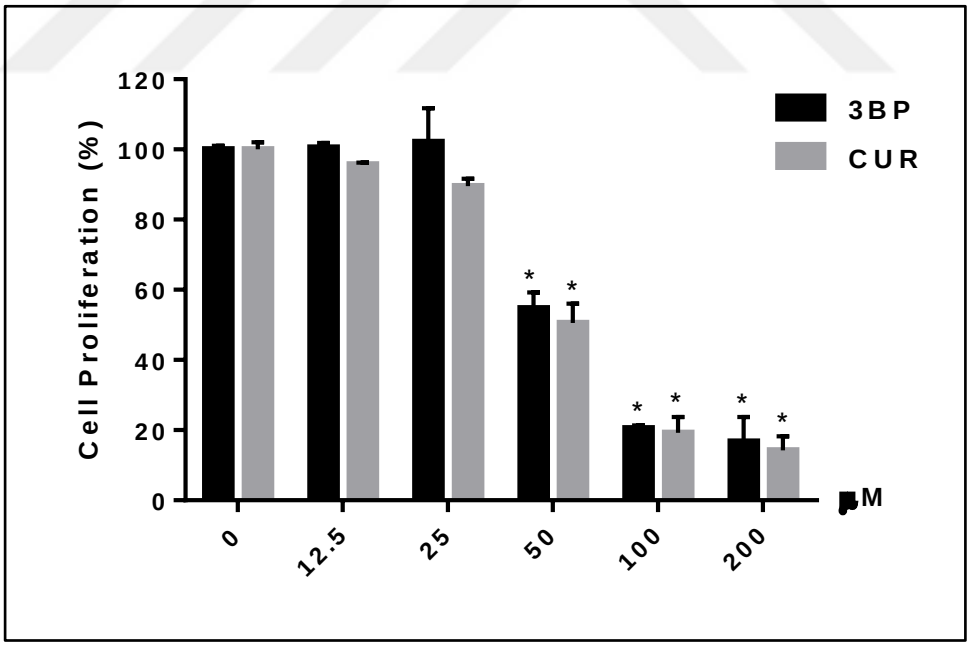

Figure 3.1. Effect of 3BP and CUR on HCT116 cell line after 24h exposure. The control group was taken as 100%. 3BP: 3-bromopyruvic acid, CUR: Curcumin. Statistical differences were showed for control vs groups as *P<0.0001. The data are expressed as mean ± SD.

Cell viability results of 3BP and CUR alone and COT treatment were shown in Figure 3.2. According to the results, COT treatment decreased the cell viability to 32 percent when compared to the 3BP and CUR groups (P<0.0001). According to the results of MTS cell proliferation assay of HCT116 cells, the combination of 3BP and COT has been found to have a greater effect on inhibition of cell proliferation with a viability of 32 percent and was also found to be parallel with the observation of the presence of fewer living cells after 24 hours in light microscopy observations.

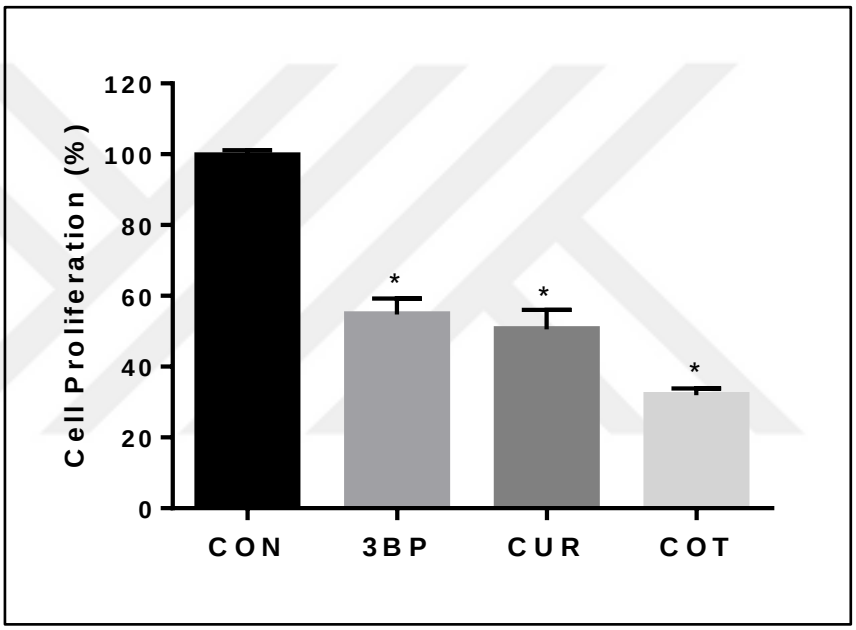

Figure 3.2. Cellular viability of HCT116 cells upon treatment with combination of 3BP and CUR for 24h exposure. Nontreated control group was taken as 100%. CON: Control, 3BP: 50 µM 3-bromopyruvic acid, CUR: 50 µM Curcumin, COT: 50 µM 3BP + 50 µM CUR. Statistical differences were showed for control vs. groups as *P<0.0001. The data are expressed as mean ± SD.

3.2. MICROSCOPICAL OBSERVATION

After treatment of HCT116 cells with 3BP, CUR, and COT, microscopical analysis was performed at 0 and 24 hours to observe cell death and morphological changes in the cells. The images were taken under 10x and 40x objectives by using brightfield microscopy. In the 3BP treatment group, cell proliferation did not increase when it is compared to the control group confluency after 24 h. When CUR and COT treatment groups were compared to the control group and also 3BP group, there was sharp decrease in cell proliferation (Figure 3.3). When morphologies of HCT116 cells were observed in more detailed objective (40x), control cells have their regular shapes and confluency after 24 h. In 3BP treatment group, cells started to change their morphology and have thin cellular extensions around them. In CUR and COT treatment groups, cells started to shrink and lose their regular shapes while they leak metabolic wastes to their extracellular matrix. In COT group, there were more single and rounded cell structures compared to the CUR group after 24 h.

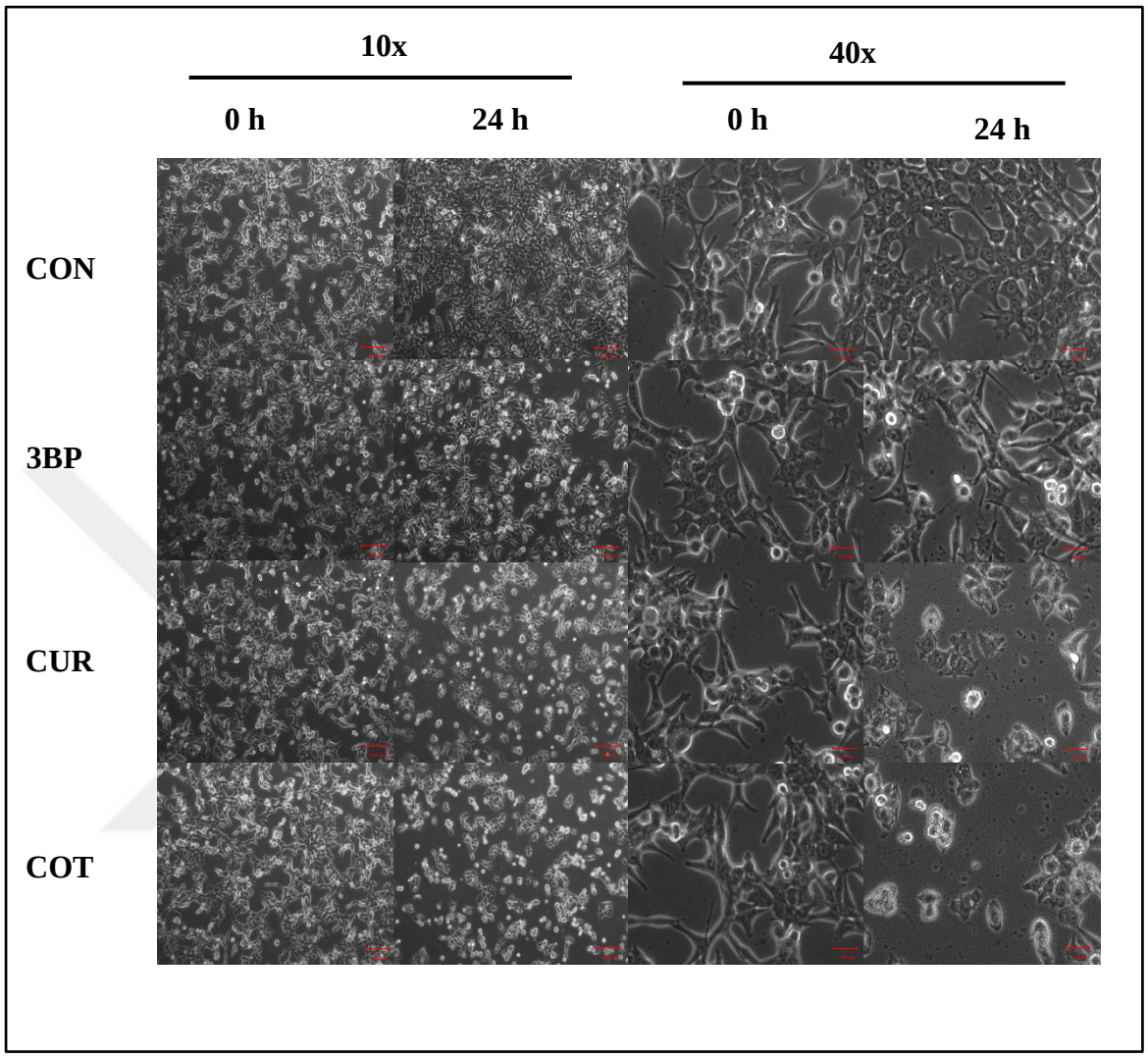

Figure 3.3. Observation of HCT116 cell morphology under light microscopy. Cells were treated with 3BP, CUR, and COT and recorded with 10x and 40x objectives after 24h.

3.3. ANNEXIN V-PI STAINING

According to the results obtained in MTS cell proliferation assay, it was revealed that COT group caused a significant amount of cell death in HCT116 cells. In accordance with these results, cell staining was performed with Annexin V-FITC Apoptosis Staining / Detection Kit in order to further determine the cause of cell death mechanism and then quantify it. For this purpose, flow cytometric analysis was conducted after 3BP, CUR, and COT treatments on cell death mechanism. After treatments, HCT116 colorectal cancer cells were stained by using Annexin V-PI dye. The four quadrants in the Fig.3.4A represents different stained cell groups. Lower left quadrant shows unstained viable cells, lower right quadrant shows early apoptotic cells which were only Annexin V stained, higher right quadrant shows late apoptotic cells which were both Annexin V and PI stained and higher left quadrant shows necrotic or dead cells which were only PI stained. According to the results, 3BP caused 35 percent early and 19 percent late apoptosis (Fig.3.4A and B). Fig.3.4A and B show, there was an induction of 99 percent late apoptosis in both CUR and COT groups which correlated obtained results in MTS cell proliferation assay and microscopic data.

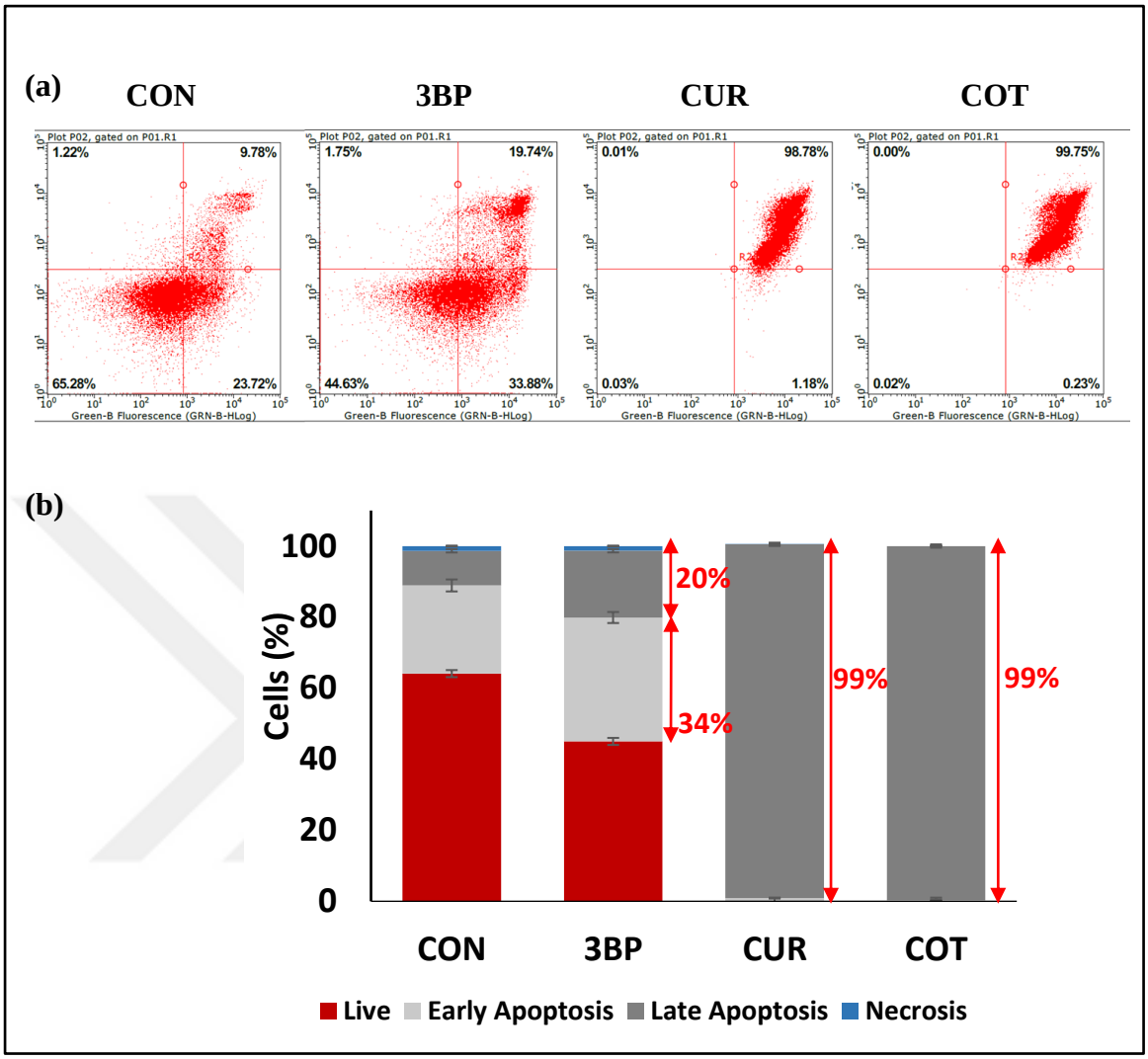

Figure 3.1. Analysis of cell death mechanism on HCT116 cells after 3BP, CUR, and COT exposure. (a) Analysis of cell death by flow cytometry. Lower right: late-apoptotic cells, upper right: early-apoptotic cells, upper left: necrotic cells, (b) Percentage of live, apoptotic and necrotic cell deaths.

3.4. INTRACELLULAR ROS MEASUREMENT

In the physiological state of normal cells, ROS plays a crucial role in maintaining homeostasis and cell signaling. When in oxidative stress conditions, ROS may trigger apoptosis by intrinsic or extrinsix pathways [75]. For this purpose, intracellular ROS levels of HCT116 cells were detected by using Intracellular ROS Detection Kit by using flow cytometry analysis. Changes in intracellular ROS levels were detected after 3BP, CUR, and COT treatments in HCT116 cells. CUR group revealed a statistically significant 50 percent increase in ROS production in HCT116 cells (Figure 3.5). In particular, COT group showed the highest intracellular ROS levels (90 percent) when it is compared to 3BP and CUR groups.

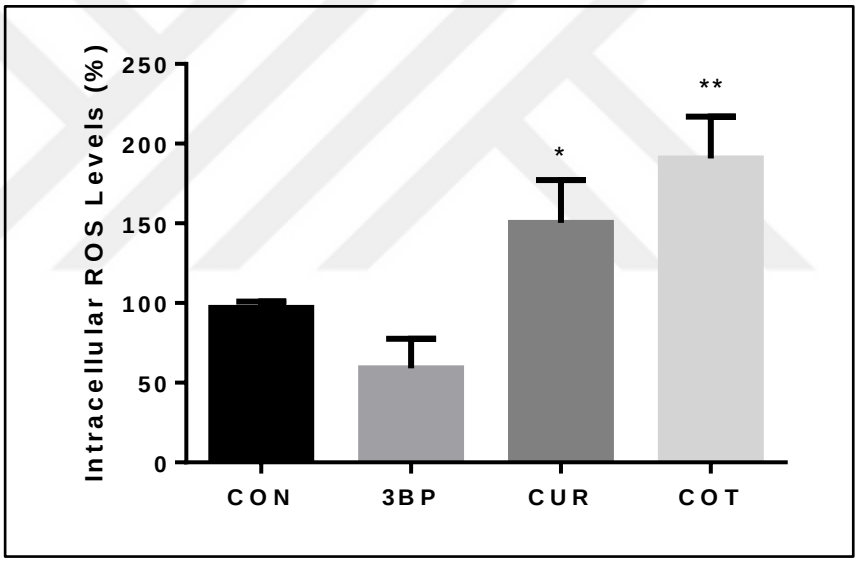

Figure 3.2. Intracellular ROS levels of HCT116 cells after 3BP, CUR, and COT. The control group was taken as 100%. Statistical differences were showed for control vs. groups as *P<0.01 and **P<0.001. The data are expressed as mean ± SD.

3.5. IMMUNOBLOTTING

3.5.1. Alterations in OXPHOS Complexes

OXPHOS protein complexes were investigated to determine the cause of excessive ROS accumulation in cells and also to reveal how mitochondrial bioenergetic was affected. For this purpose, immunoblotting experiment was performed with OXPHOS Rodent WB Antibody Cocktail to investigate protein expression changes in OXPHOS protein complexes after 3BP, CUR, and COT. According to the results, Figure 3.6 shows that no substantial protein expression change upon 3BP or CUR alone on HCT116 colorectal cells. However, when cells were treated with 3BP and CUR in combination (COT), Complex III and Complex II protein expression levels were significantly decreased by 21 and 32 percent, respectively. Immunoblotting results of OXPHOS protein complexes indicate functional impairment in Complex II and III with COT on HCT116 colorectal cancer cells.

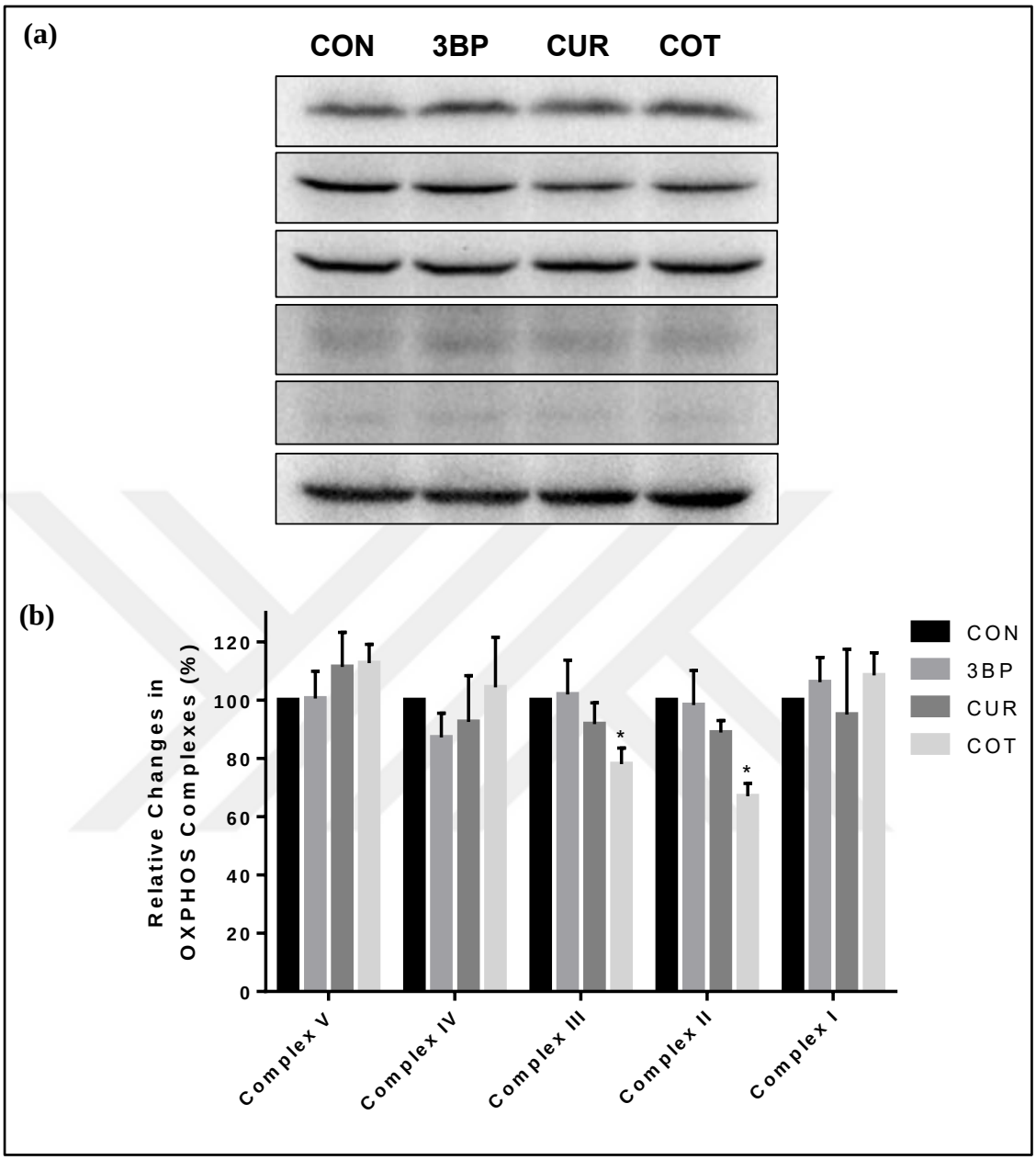

Figure 3.3. Alterations of OXPHOS complexes on HCT-116 cells after exposure of 3BP, CUR, and COT. Nontreated control group was taken as 100%. Complex V: ATP synthase, Complex IV: Cytochrome c oxidase, Complex III; Cytochrome bc1 complex, Complex II: Succinate dehydrogenase, Complex I: NADH dehydrogenase. Statistical differences were showed for control vs. groups as *P<0.01. (a) Immunoblotting analysis of OXPHOS complexes and B-actin, (b) Relative changes in OXPHOS complexes (%).

3.5.2. Alterations in LDH-A Level

LDHA is an NAD^+-dependent enzyme that catalyzes pyruvate to lactate in the last step of glycolysis pathway. It has been stated in previous studies that cancer cells showing high malignancy with Warburg effect have an upregulation especially in colorectal cancer and thus promote aerobic glycolysis in cells [90]. For this purpose, alterations in the protein expression levels of LDH-A enzyme on HCT116 colorectal cancer cells upon 3BP, CUR, and COT were investigated by immunoblotting experiments. According to the results shown in the Figure 3.7, immunoblotting analysis of LDH-A levels were demonstrated that COT significantly decreased protein expression levels of LDH-A by 36 percent when its compared to untreated control ($P<0.01$). Obtained data from LDH-A immunoblotting point out that COT significantly suppressed glycolysis pathway in HCT116 colorectal cancer cells.

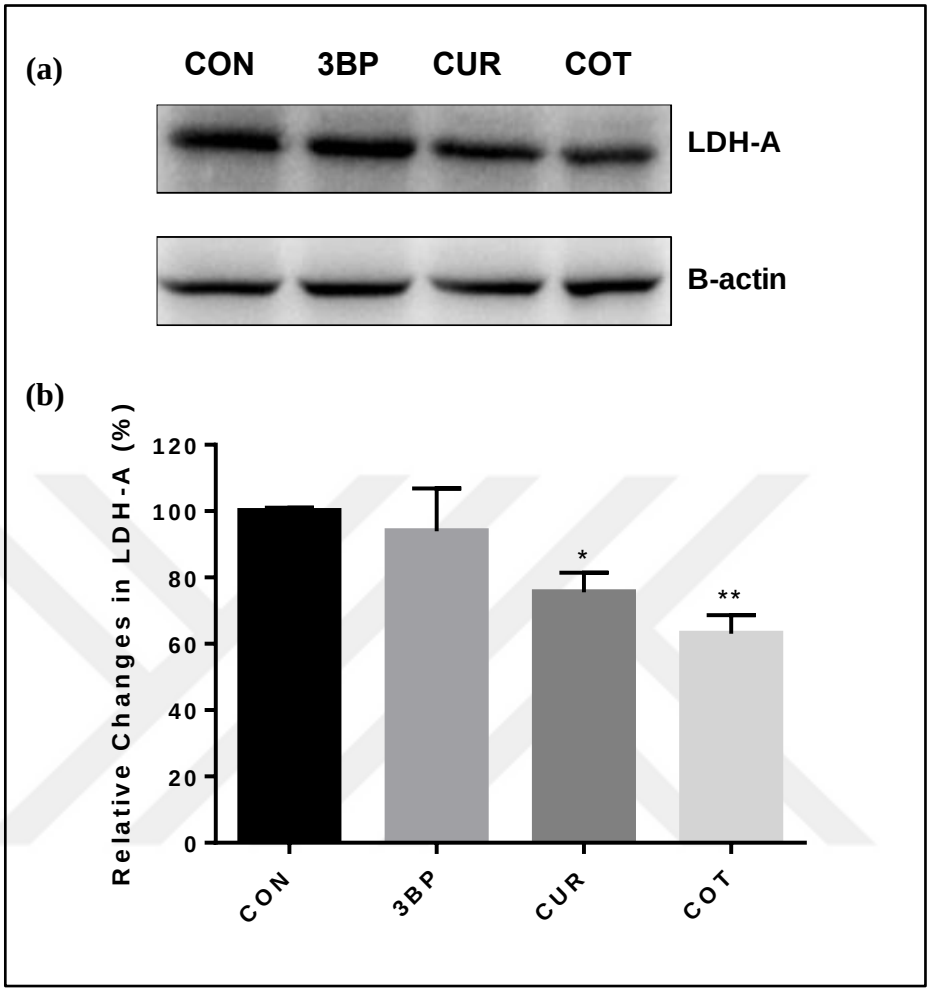

Figure 3.4. Alterations of LDH-A levels on HCT-116 cells after exposure of 3BP, CUR, and COT. Nontreated control group was taken as 100%. LDH-A: Lactate dehydrogenase-A. Statistical differences were showed for control vs. groups as **P*<0.05, ***P*<0.01. (a) Immunoblotting analysis of LDH-A and B-actin, (b) Relative changes in LDH-A (%).

3.6. CONFOCAL IMAGING

3.6.1. Mitotracker Green FM Staining

HCT116 cells stained with Mitotracker Green FM dye were observed by confocal microscopy technique in order to observe alterations in mitochondrial mass as well as physiological changes in mitochondria induced by 3BP, CUR, and COT. Mitochondrial mass content was measured in colorectal cancer cells after the exposure of 3BP, CUR, and COT for 24h. Intensities were measured and calculated by using ZEN 3.0 Blue Edition (Carl Zeiss Microscopy, Germany). According to the confocal imaging results, mitochondrial mass content was decreased by 3BP, CUR, and COT groups when they are compared with CON (Figure 3.8). Especially in the COT group, mitochondrial mass content was decreased to 35 percent while 3BP and CUR groups decreased mass content to 72 and 60 percent, respectively. Imaging results exhibited that COT reduced healthy mitochondria content and distrupts its physiology in HCT116 colorectal cancer cells.

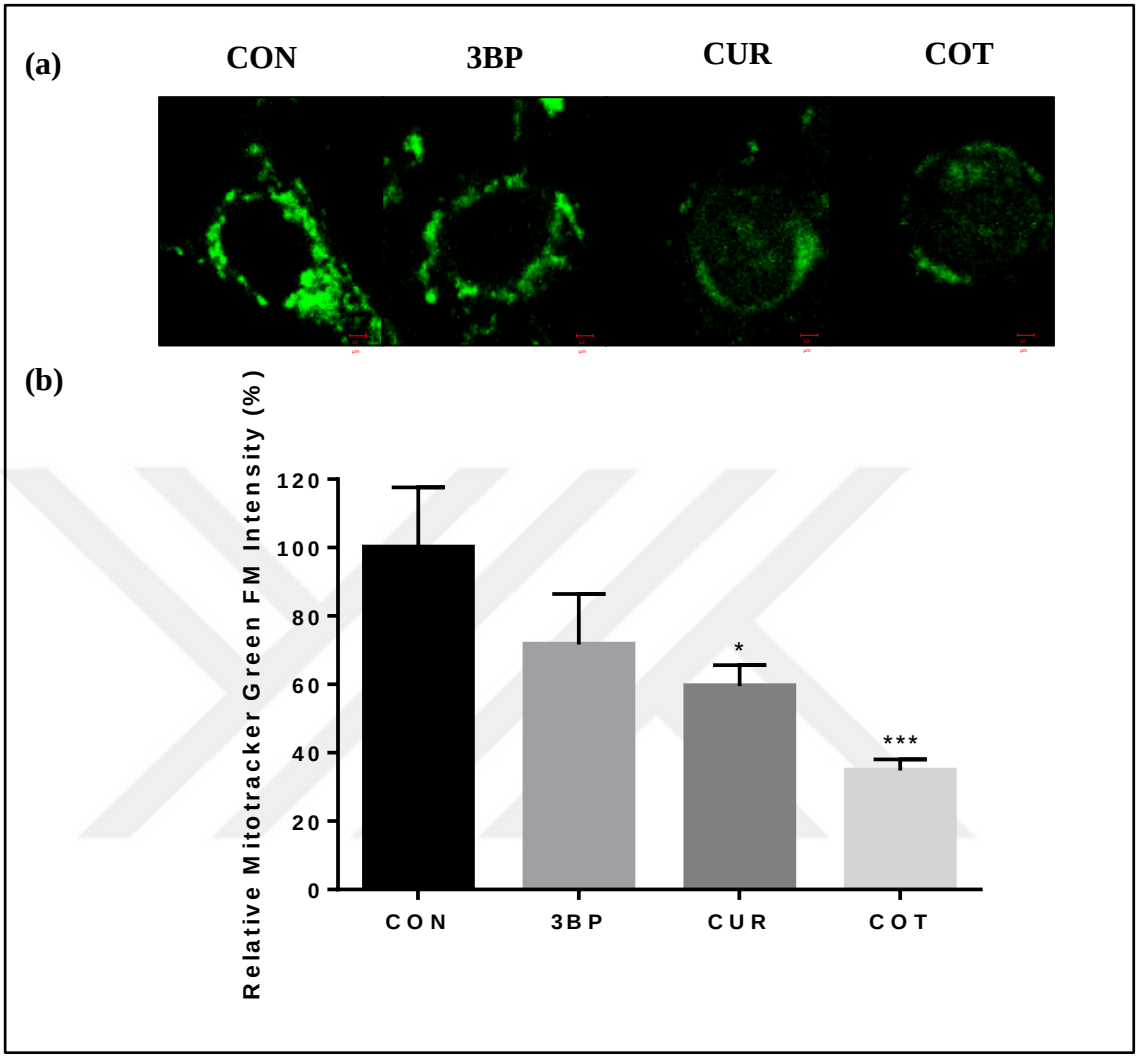

Figure 3.5. Mitochondrial mass content analysis of HCT-116 cells after exposure of 3BP, CUR, and COT. Nontreated control group was taken as 100%. Statistical differences were showed for control vs. groups as *P<0.05, ***P<0.001. (a) Mitotracker Green FM staining of HCT116 cells, (b) Relative Mitotracker Green FM intensity changes (%).

3.6.2. Rhodamine 123 Staining

The mitochondrial membrane potential ($\Delta\Psi m$) is created by four different protein complexes (Complex I, II, III, and IV) located in the inner membrane of the mitochondria [91]. According to previous studies, oxidative stress caused by ROS accumulation triggers apoptosis, as well as causing dysregulation in the cell mitochondria and subsequently causing loss of inner membrane potential [92]. Rhodamine 123 was used to measure inner mitochondrial membrane potential (MMP, $\Delta\Psi_M$) of HCT116 cell line after treatment with 3BP, CUR, and COT by confocal microscopy technique. Intensities were measured and calculated by using ZEN 3.0 Blue Edition (Carl Zeiss Microscopy, Germany). According to the results, all treatment groups lead to a significant decrease in mitochondrial membrane potential (Figure 3.9). Especially in the COT group, mitochondrial membrane potential was decreased to 36 percent while 3BP and CUR groups decreased membrane potential to 62 and 52 percent, respectively. MMP loss was demonstrated that COT significantly disrupts proton gradient across inner membrane of mitochondria.

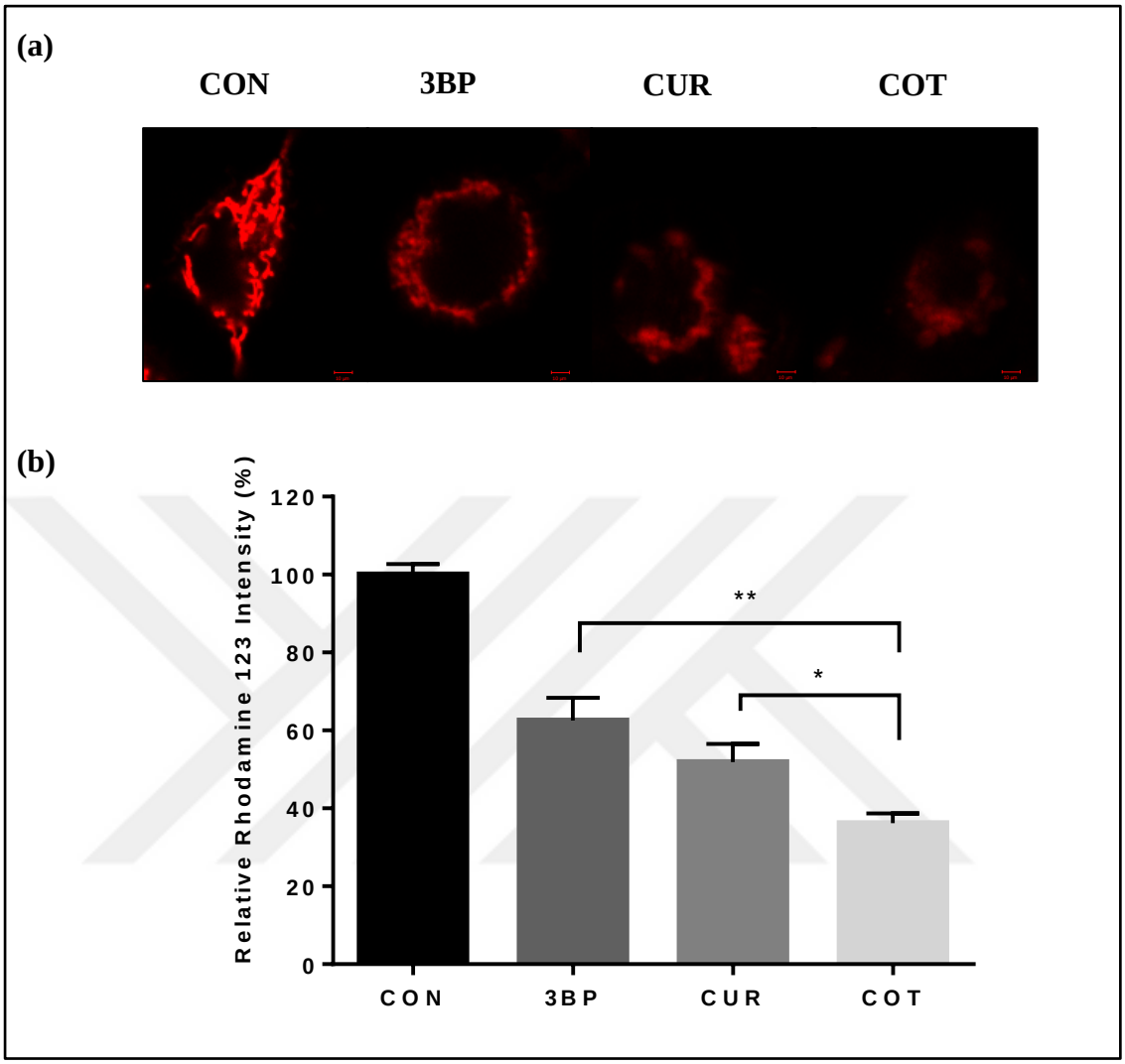

Figure 3.6. Mitochondrial membrane potential of HCT-116 cells after exposure of 3BP, CUR, and COT. Nontreated control group was taken as 100%. Statistical differences were showed for COT vs. groups as *P<0.05, **P<0.01. (a) Rhodamine 123 staining of HCT116 cells, (b) Relative Rhodamine 123 intensity changes (%).

4. DISCUSSION

Most of the cancer cells depends on aerobic glycolysis that commonly stated as ATP production by employing glycolytic metabolism even in the presence of oxygen [12]. It is a metabolic switch in a hypoxic environment and the resulting acidosis has been associated with promoting tumorigenesis and metastasis, becoming an advantage for cancer cells [18]. Various glycolytic enzyme inhibitors that target key steps in the pathway have been studied in recent years [93]. Among these, one of the commonly studied inhibitors is 3-bromopyruvate (3BP) which showed its activity in both *in vivo* and *in vitro* studies [94]. It is a pyruvate analog that exhibits its anti-cancer activity mainly by inhibiting hexokinase II (HKII), thus blocking glucose entrance for the glycolysis pathway. There is increasing attention focusing on nutraceuticals as a potential therapy for cancer by having less toxicity to healthy cells and also trackable side effects. Curcumin which is the active component of *Curcuma longa* has been intensively studied for its anti-cancer effect [62]. It has been demonstrated that it induces cell death, ROS production, and apoptosis [95]. Combination therapies, which increase the patient's quality of life by aiming to advance the treatment or weaken the side effects instead of the conventional therapies currently used, have recently become very important Traditionally used chemotherapeutic agents cause painful side effects in most patients. In many studies, many pathways can be targeted with combination therapy compared to conventional chemotherapeutic and it has been shown that using drugs at lower doses leads to more effective cell death [96]. Combination therapies also accelerate drug resistance defeat, and there is no need for new drug discovery by combining existing drugs [97,98]. According to the analyzes for drug development processes in the therapeutic area, it has been stated that it takes at least 12 years or even more years to determine the target of a drug, then start research and get approval in the market [99].

In our study, the aim is to reveal the combinatory effects of 3BP and CUR on cellular, mitochondrial, and glycolytic metabolism of HCT116, colorectal cancer cell line. In this aspect, firstly single drug optimal effective concentrations for each of 3BP and CUR were determined before deciding combination treatment (COT) (Figure 3.2). A recent finding of Bodur *et al*., (2016) showed that cell proliferation of HCT116 cells inhibited by 30 percent by treatment with 50 μM 3BP for 24h [100]. According to our proliferation study, 24h treatment of 50 μM 3BP decreased cell viability to 55 percent. A previous finding of Lim *et*

al. (2014) showed that 50 μM CUR suppressed cell proliferation of HCT116 cells by 40 percent for 24h treatment [81]. Similarly in our study, and 50 μM CUR decreased cell viability to 51 percent which were found as parallel to the previous literature findings. After determining combination concentrations of 3BP and CUR which were 50 μM for both drugs, cell proliferation analysis showed COT enhanced the inhibition of cell proliferation with a viability of 32 percent on colorectal cancer cells after 24h treatment (Figure 3.3). The enhancement in the inhibition of cell proliferation after COT may be associated with 3BP and CUR combination effects targeting multiple pathways in the cell death mechanism.

Inhibition of proliferation obtained in MTS cell proliferation assay results, as well as parallel to MTS assay, with the observation of morphological disruption of cells under the microscope, Annexin V-PI staining experiment was performed to investigate and quantify cell death by using flow cytometric analysis. According to previous studies, Chen *et al*. (2009) showed that 3BP causes dissociation of HKII enzyme from mitochondria thus releases of apoptosis-inducing factor (AIF) to the cytosol and eventually apoptosis [94]. Similary, Ho *et al*. (2016) revealed that 50 μM 3BP treatment caused 55 percent late apoptosis on HCT116 cells [49]. On the other hand, several studies have shown that curcumin efficiently induces late apoptosis in various cancer types by promoting the release of cytochrome *c* into cytosol through initiating mitochondrial-mediated intrinsic apoptotic pathway, including melanoma [101], prostate [102], breast [103], colorectal [104]. In contrast to the antioxidant activity shown by curcumin at low doses, it was stated that it showed pro-apoptotic effects on many types of cancer at high concentration. In the study of Collett *et al*., 2004, after curcumin application on HCT116 colorectal cancer cell line, apoptotic cell death was observed depending on the dose, followed by c-jun N-terminal kinase (JNK) and p38 mitogen activated kinase (MAPK) activation along with suppression was observed in transcription activity of nuclear factor-kappa B (NF-κB) [105]. According to our results of Annexin V-PI analysis, CUR and COT doubled the late apoptotic cell number (99 percent) in HCT116 cells which revealed that late apoptosis is involved in cell proliferation inhibition of CUR and COT (Figure 3.4). Apoptosis, which is one of the cell death mechanisms, is a type of energy-dependent death. Besides, it is a programmed and controlled cell death mechanism, as well as a process that does not cause inflammation and environmental damage [106]. When cells are exposed to stress or injuries from the environment such as anti-cancer therapeutics, UV radiation, DNA damage, starvation,

endoplasmic reticulum (ER) stress, excessive ROS stress, induce cell death by triggering mechanisms of apoptotic cell death [107]. Cells showing apoptotic cell characteristics show physical and biochemical markers such as blebbing in the plasma membrane, translocation of phosphatidylserine, cell deatachment, mitochondrial membrane permeability, and release of mitochondrial content, DNA fragmentation [106]. On the other hand, necrotic cell death is classified as an uncontrolled and accidental cell death, and it is seen as the collapse of the cell membrane and organelles due to factors such as osmotic stress, temperature, collapse, and extreme pshysiochemical stress. Therefore, it is characterized as a type of death that causes a local inflammation along with intracellular factors that the dead cells release [108]. According to the results obtained in our study, the fact that the death mechanism originates from late apoptosis is a preferred mechanism of death since it is a mechanism that does not cause inflammation in the environment.

Since ROS accumulation at excessive amounts has been shown to trigger the apoptotic death mechanism via intrinsic and extrinsic pathways, it was aimed to investigate intracellular ROS levels in colorectal cancer cells. ROS are chemically reactive molecules that are mainly produced in the mitochondria which are included in numerous important physiological and pathological pathways within the cell [109]. However, functional impairments and leaks in electron chain complexes (ETC) in mitochondria that has major role in ROS production, thus lead to ROS accumulation in cells [93]. Curcumin is a well known potent antioxidant in normal cells by scavenging low levels of ROS [54]. On the contrary, CUR acts as a pro-oxidant molecule at high doses by inducing excessive ROS accumulation to serve as a pro-apoptotic molecule by triggering cytochrome c release and eventually mitochondria-mediated apoptosis on colorectal cancer cells [110]. Previous studies showed that curcumin may lead to dysfunctions in antioxidant defense system including depletion in non-enzymatic antioxidant glutathione (GSH) levels [111]. Also, it indirectly regulates the expression of important antioxidant enzymes including SOD, GPX, and HO-1 [112]. It is also well-known that glycolysis-dependent cancer cells showing the Warburg effect causes elevated ROS levels which plays an important role in cancer progression. However, since ROS levels in cancer cells are elevated and they are closer to cell-death threshold, ROS triggering stimuli cause cell death by overwhelming antioxidant systems more [75]. Therefore, ROS-induced oxidative stress formation has become an effective strategy for inhibiting cancer cell formation or proliferation in recent research studies [94]. In this study,

ROS levels were investigated by using DCFDA Intracellular ROS Detection Assay upon 3BP, CUR, and COT. According to the results, COT significantly increased intracellular ROS levels to 90 percent on HCT116 cells when it is compared to control (P<0.001) (Figure 3.5). However, there was no significant increase in ROS levels in cells exposed to 3BP. However, according to the result of protein expression analysis in OXPHOS complexes, no change in ROS levels may be due to the lack of any significant change in the expression of the complexes due to 3BP exposure. In addition, mitochondrial uncoupling protein-2 (UCP2) is a protein found in the inner membrane of the mitochondria and is an important protein in the first line of the antioxidant defense system involved in the elimination of mitochondrial ROS [113]. In studies on colorectal cancer, UCP2 has been found to be upregulated, and this may be an adaptation to escape the apoptotic death mechanism that may result from oxidative stress, and also to provide cells with chemoresistance [114]. Therefore, UCP2 expression levels might be examined to understand if COT has an effect on this system that may be a cause of ROS accumulation and following cell death.

The inner mitochondrial membrane potential (MMP ($\Delta\Psi_M$)) is crucial for the survival of the cell by maintaining normal cellular function and physiology. The inner mitochondrial membrane potential loss is known to be one of the causes of leading cells to mitochondria-initiated intrinsic apoptotic death [115]. In this study, mitochondria mass content was investigated to further understand underlying cell death mechanisms by using MitoTracker Green FM dye. Stained cells were analyzed with confocal imaging in order to observe mitochondrial structure and function of cells after 3BP, CUR, and COT treatments. According to the results, COT treatment causes significant decrease of 35 percent in mitochondrial content in HCT116 cells (Figure 3.8). In parallel, in order to investigate MMP ($\Delta\Psi_M$) in cells, Rhodamine 123 dye was used. It has been shown in previous studies that the 3BP molecule causes the HKII enzyme to dissociate from the outermembrane of mitochondria [49]**.** Interaction of HKII with VDAC is important for preventing the formation of mitochondrial permeability pores and ensuring mitochondrial integrity [37,116]. Ihrlund *et al.* (2008) showed that inner mitochondrial membrane potential decreased by half with 100 μM 3BP in HCT116 cells [45]. On the other hand, previous findings of Su *et al*., was revealed that 50 μM curcumin lead intrinsic apoptotic pathway by inducing ROS accumulation followed by 50 percent MMP ($\Delta\Psi_M$) decrease, induces the releasing of cytochrome c and activation BAX and caspase 3 [110]. According to obtained results, COT

lead to 64 percent decrease in MMP levels which indicates 3BP and CUR combination enhanced dissipation of MMP in HCT116 cells. Subsequently, it is reasonable to speculate that the decrease in mitochondrial membrane integrity after 3BP, CUR, and their combination may be related to HKII dissociation from mitochondria (Figure 3.9). Whether the interaction of HKII with VDAC has disappeared may be examined to further clarify the mechanism. VDACs also is crucial for controlling the passage of metabolites into mitochondria, through providing cross-talk between mitochondria and the entire cell [117]. Therefore, with COT application, it can be studied whether the connection between the cell and the mitochondria has disappeared and caused impairment in the metabolic and energetic functions of the mitochondria.

Since mitochondria have a major role in ROS production, electron leaks resulting in functional dysfunctions of OXPHOS protein complexes and also dysfunctions in the antioxidant defense system lead to ROS accumulation in cells [118]. For this purpose, OXPHOS complexes were analyzed if the oxidative stress is caused by dysfunctions in complexes. Levels of protein expressions in OXHPOS complexes were investigated by immunoblotting experiment after 3BP, CUR, and COT on HCT116 cells. According to the results obtained, COT was found to cause 32 percent decrease in Complex II (succinate dehydrogenase, SDH) which presents the subunit B of complex II (SDHB). In addition, it was observed that 21 percent decrease in protein expressions levels of Complex III (cytochrome bc1) which presents the subunit 2 of Complex II (UQCRC2) (Figure 3.6). Complex II is vital for cell metabolism by taking part in the TCA cycle and especially in the formation of hydrogen peroxide (H_2O_2) and superoxide radicals. In studies using anti-cancer drugs that cause functional disruptions in Complex II, it was observed that ROS production increased due to a large number of electrons leak to molecular oxygen and thus cause cell death due to apoptosis [119]. On the other hand, Complex III is another complex where ROS formation occurs, especially superoxide anion radical (O_2•-). It is also a complex that contributes to the formation of membrane potential and proton gradient. Considering all these results, it might be concluded that the changes occurring in the OXPHOS complexes lead to the formation of ROS by electron leaks followed by distruptions in integrity of mitochondria and apoptotic cell death.

In addition to investigating mitochondrial energy metabolism, another aim of our study was to reduce the energy production pathway by targeting the glycolysis pathway. It has been

shown in previous studies that both 3BP and CUR have reduced lactate dehydrogenase-A (LDHA) protein expression and enzyme activity [120,121]. LDHA is an NAD^+-dependent enzyme that catalyzes pyruvate to lactate in the last step of glycolysis pathway [122]. It is correlated with malignant cancer types with glycolysis-dependent aggressive phenotypes that show Warburg effect, particularly colorectal cancer [90,122]. LDHA plays a key role in cells producing ATP with aerobic glycolysis and maintaining their proliferation, even at low oxygen levels [123]. Therefore, targeting the LDHA enzyme and such glycolysis targeting molecules in cancer treatments has started to gather attention in recent studies [123]. For this purpose, LDH-A enzyme protein expression levels were investigated to monitor glycolytic metabolism by immunoblotting experiment. In our results, the expression of LDH-A was downregulated (36 percent) in HCT116 cells upon treatment with 3BP and curcumin together compared to control cells (Figure 3.7). Therefore, 3BP and CUR together suppressed glycolysis-dependent energy metabolism in colorectal cancer cells by reducing the expression of a key glycolytic enzyme which facilitates lactate production. For further investigation of glycolysis, extracellular acidification rate (ECAR) through lactate production upon COT should be analyzed by using Seahorse XF96 Extracellular Flux Analyzer.

As a summary, the combination of 3BP and CUR applied on HCT116 colorectal cancer has been found to inhibit both the mitochondrial and glycolytic energy production pathways by inhibiting the proliferation of cells, providing a controlled and clean form of apoptotic death and triggering oxidative stress.

5. CONCLUSION AND FUTURE PERSPECTIVE

In conclusion, this study demonstrates that 3BP and CUR combination (COT) is more effective than single drugs on HCT116, colorectal cancer cell line by altering both glycolysis and OXPHOS bioenergetic pathways. The combination of 3BP and CUR leads to enhanced cell proliferation inhibition which is caused by late apoptosis. Further cellular metabolism studies showed that COT significantly increased intracellular ROS production level, which may alter mitochondrial function and metabolism. Later, mitochondrial metabolism studies indicated that COT strongly disrupted mitochondrial content, function, and inner mitochondrial membrane potential. Also, protein expression levels of OXPHOS complexes including Complex II (succinate dehydrogenase) and Complex III (cytochrome bc1) were decreased with COT which may lead to ROS accumulation in cells. Finally, glycolytic metabolism studies revealed that COT significantly suppressed the expression of LDH-A, which alters the energy metabolism of cells.

Taken all together, our results propose that the 3BP and CUR combination, COT, might be a new option for both preventing and treating colorectal cancer which needed further studies to enlighten its mechanism of action. Firstly, COT should be applied to normal colorectal cells to understand its effects. The interaction of HKII with VDAC may be examined to further elucidate the mitochondrial alterations mechanism by performing immunoblotting experiment from mitochondria and cytosolic fractions. In order to further investigate alterations in antioxidant defense system NRF2 related enzymes SOD, GPX, HO-1, and non-enzymatic antioxidant GSH may be studied. Also UCP2 expression might be analyze to check if it is involved in ROS accumulation mechanism. Underlying intrinsic apoptosis mechanism should be studied including caspase 3 activity and cytochrome c release. C-jun N-terminal kinase (JNK) and p38 mitogen activated kinase (MAPK) related apoptotic cell death mechanism and also nuclear factor-kappa B (NF-κB) transcriptional activity might be investigated [105]. Cyclooxygenase-2 (COX-2) is involved in the conversion of arachidonic acid to prostaglandins and play a role in regulating apoptosis and cell proliferation in cancer cells. In many *in vivo* and *in vitro* studies, the COX2 enzyme has been shown to be upregulated in colorectal cancer. Colorectal cancer cells with COX2 overexpression tend to escape the apoptotic death mechanism, by reducing cytochrome *c* release, inhibition of caspase activation, and controling anti-apoptotic protein Bcl-2 upregulation [124]. Based on

this, protein expression of COX2 would be examined. Extracellular acidification rates (ECAR), which is basically affected by lactate production in cells, might be analyzed by using Seahorse XF96 Extracellular Flux Analyzer in order to unravel glycolytic rate decrease related with our results. In the metabolic pathway proteome analysis on colorectal cancer by Bi *et al*., 2006 showed upregulation in glycolytic enzymes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and enolase 1. Also, alterations in aconitase and aconitate hydratase were demonstrated which cause impairment in TCA [125]. These enzymes may be examined with 2-dimensional gel electrophoresis (2-DE) coupled mass spectrometry upon COT. Results obtained from cellular, mitochondrial, and glycolytic metabolism experiments may offer preliminary knowledge for future *in vitro* and *in vivo* studies.

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