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YEDİTEPE UNIVERSITY

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THE EFFECT OF MELATONIN ON CHEMOTHERAPEUTIC ACTIVITY OF PACLITAXEL ON HCT-116-HUMAN COLON CELL LINE

MASTER THESIS

Yasmine AL HASSADI

SUPERVISOR

Associate Prof. Burcu GEMICI BASOL

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APPROVAL

This thesis has been deemed by the jury in accoidance with the relevant articles of Yeditepe Univelsity Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated **17/05/2019** and numbered **2019/08-37**

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgement has been made in the text.

Date: 1. Feb. 2019

Name Surname

Yasmine AL HASSADI

BEYAN

Bu tezin kendi çalışmam olduğunu, planlanmasından yazımına kadar hiçbir aşamasında etik dışı davranışımın olmadığını, tezdeki bütün bilgileri akademik ve etik kurallar içinde elde ettiğimi, tez çalışmasıyla elde edilmeyen bütün bilgi ve yorumlara kaynak gösterdiğimi ve bu kaynakları kaynaklar listesine aldığımı, tez çalışması ve yazımı sırasında patent ve telif haklarını ihlal edici bir davranışımın olmadığını beyan ederim.

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Adı Soyadı

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LIST OF SYMBOLS AND ABBREVIATIONS

AA-NAT	Arylakylamine N-acetyltransferase
AFMK	*N*-acetyl-formylo-5-metoxykynuramine
AMK	*N*-acetyl-5-metoxykykuramine
APC	Adenomatous Polyposis Coli (APC) tumor suppressor gene
BSP	Bone sialoprotein
cAMP	Cyclic adenosine monophosphate
caMKII	Ca2+/calmodulin-dependent protein kinase IIα
CAT	Catalase
CIMP	CpG island methylator phenotype
CIN	Chromosomal instability
CK1	Casein kinase 1
CRC	Colorectal cancer
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's modified eagle medium
DPBS	Dulbecco's phosphate buffered saline
ECM	Extracellular matrix
EGF	Epidermal growth factor
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin-1
FAP	Familial adenomatous polyposis
FBS	Fetal bovine serum
G -protein	Guanine nucleotide binding protein
GDP	Guanosine diphosphate
GEF	Guanine exchange factors
GSH	Glutathione reductase
GSK3	Glycogen synthase kinase 3
GPx	Glutathione peroxidase
GRB2	Growth-factor-receptor bound protein 2

GTP	Guanosine triphosphate
HDCA4	Histone deacetylase 4
HNPCC	Hereditry non-polyposis colorectal cancer
13-HODE	Hydroxy octadecadienoic acid
H2O2	Hydrogen superoxide
HPS	Hyperplastic polyposis syndrome
5-HTP	5-hydroxytryptophan
IL	Interleukin
IML	Intermediolateral nucleus
K-RAS	Kirsten rat sarcoma gene
LA	Linoelic Acid
LRP6	Low-density lipoprotein receptor-related protein 6
LOH	Loss of heterozygosity
5-LOX	5-lipoxygenase
MAPs	Microtubulin associated proteins
MAPK	Mitogen-activated protein kinase
MMR	Mismatch repair
MSI	Microsatellite instability
aMT6s	6-sulfatoxymelatonin
MTN	Melatonin
NAS	N-acetylserotonin
NE	Norepinephrine
OH	Hydroxyl radicals
O2-	Superoxide anionic radical
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PIK3CA	Type I phosphatidylinositol 3 kinases
PKc	Protein kinase C
PLc	Phospholipase C
PTX	Paclitaxel

PVN	Paraventricular neurons
QR2	Quinone reductase 2
ROS	Reactive oxygen species
SCG	Superior cervical ganglion
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SSA	Sessile serrated adenoma
SOD	Superoxide dismutase
TCF/LEF	T cell factor/lymphoid enhancer factor
TGF	Tissue growth factor
TH	Tryptophan hydroxylase
Th	T helper cells
TNF- α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

ABSTRACT

Al Hassadi, Y. (2019). The Effect of Melatonin on Chemotherapeutic Activity of Paclitaxel on HCT-116-Human Colon Cell Line. Yeditepe University, Institute of Health Science, Department of Physiology, MSc Thesis, İstanbul.

Colorectal cancer is one of the most prevalent cancer type. Combinatorial treatment strategies are important field of cancer research. Paclitaxel (PTX) is a chemotherapy medication used to treat huge number of types of cancer. However, the resistance to the PTX treatment restrict its use in the clinics. Melatonin (MTN) is mainly produced by pineal gland. Besides its well-known function as the regulation of the circadian rhythm, anticancer effects have been indicated. The aim of this study is to determine the effect of MTN on PTX treatment.

In our study, human colorectal carcinoma cell line, HCT-116 was used. The toxicity, the dose and the duration of exposure of PTX and MTN were determined using the water-soluble tetrazolium-1 (WST-1) colorimetric cell proliferation assay kit. HCT-116 cells were treated with different combinations of MTN, PTX and MTN + PTX for 24 hours. Apoptosis and necrosis findings were obtained by flow cytometry using MEBCYTO Apoptosis Kit. Statistical analysis was performed using Kruskal Wallis and Mann Whitney U tests with helping of SPSS 18 software program.

Five different doses and two different exposure times were tried for MTN and PXL, and the dose of MTN was 500 μM and PXL was 50 nM ($p < 0.05$). Exposure time is 24 hours for both substances. When the increasing doses of MTN were applied together with PXL, it was observed that the cell viability decreased compared to the group treated with PXL ($p < 0.05$). While 50 nM and 24 hour PXL administration increased apoptosis, addition of 24-hour MTN to this application resulted in an increase in apotosis ($p < 0.05$). Compatible with the apoptosis data, the addition of MTN in necrosis results increased the effect of PXL ($p < 0.01$). Our results suggest that MTN potentiates the chemotherapeutic effect of PTX in a dose dependent manner.

ÖZET

Al Hassadı, Y. (2019). Melatoninin HCT-116 İnsan Kolon Hücre Hattında Paklitakselin Kemoterapötik Aktivitesine Etkisi. Yeditepe Üniversitesi Sağlık Bilimleri Enstitüsü, Fizyoloji ABD. Master Tezi. İstanbul.

Kolorektal kanser en sık görülen kanser tiplerinden biridir. Kemoterapötik ajanların kombine tedavi stratejileri, kanser araştırmalarının önemli bir alanıdır. Paklitaksel (PTX), çok sayıda kanser türünü tedavi etmek için kullanılan bir kemoterapi ilacıdır. Ancak, PTX tedavisine direnç ve değişkenlik, kliniklerde bu ilacın kullanımını kısıtlamaktadır. Epifiz bezi tarafından üretilen melatonin (MTN)'in sirkadiyen ritimin regülasyonu olarak bilinen temel işlevinin yanı sıra, çeşitli kanser türlerine karşı anti-kanser etkileri de gösterilmiştir. Bu çalışmanın amacı MTN'nin PTX'in kemoterapötik aktivitesine etkisini belirlemektir.

Çalışmamızda insan kolorektal kasinoma hücre hattı, HCT-116 kullanılmıştır. Water-soluble tetrazolium-1 (WST-1) kolorimetrik hücre proliferasyon analiz kiti kullanılarak PTX ve MTN'nin toksisitesi, etken dozu ve maruz kalma süresini belirlenmiştir. HCT-116 hücreleri, 24 saat süre ile MTN, PTX ve MTN+PTX'in farklı kombinasyonları ile muamele edilmiş, MEBCYTO Apoptosis Kit kullanılarak Flow sitometri yöntemi ile apoptoz ve nekroz bulguları elde edilmiştir İstatistiksel analiz tek yönlü Kruskal Wallis ve Mann Whitney U testleri kullanılarak SPSS 18 yazılım programı yardımı ile yapılmıştır.

MTN ve PXL için beş farklı doz ve iki farklı maruz kalma süreleri denenmiş, MTN etken dozu 500 μM, PXL etken dozu 50 nM olarak belirlenmiştir ($p<0.05$). Maruz kalma süresi her iki madde için 24 saattir. MTN'nin artan dozları PXL ile beraber uygulandığında sadece PXL uygulanan gruba göre hücre canlılığının azaldığı gözlenmiştir ($p<0.05$). 50 nM ve 24 saatlik PXL uygulaması apoptozu artırırken, bu uygulamaya 24 saatlik MTN eklenmesi apaotozda artışa sebep olmuştur ($p<0.05$). Apoptoz verileri ile uyumlu şekilde Nekroziz sonuçlarında da MTN'in eklenmesi PXL'in etkisini artırmıştır ($p<0.01$). Sonuçlarımız MTN'nin PTX'in kemoterapotik etkisini doz bağımlı bir şekilde artırdığını göstermektedir.

1. INTRODUCTION and PURPOSE

Combinational treatment strategies to improve the efficacy of the chemotherapeutic agents are of interest in the field of cancer research. Colorectal cancer is one of the most prevalent cancer type with a relatively high death incidence. Melatonin (*N*-acetyl-5-methoxytryptamine; MTN) is an endogenous indolamine mainly produced by pineal gland. Besides its well-known function as the regulation of the circadian rhythm, anti-cancer effects of MTN against various cancer types have been indicated via interfering the cancer promoting mechanisms such as promoted proliferation, metastasis and angiogenesis. Paclitaxel (PTX) is a naturally occurring anti-cancer compound and is found successful against many cancer types in the clinics. However, the acquired resistance and variability in the response of patients to the PTX treatment restrict its use in the clinics. The aim of this study is to explore the role of MTN on chemotherapeutic activity of PTX.

2. LITERATURE REVIEW

2.1. Melatonin

2.1.1. Melatonin Introduction

Melatonin hormone (N-acetyl-5-methoxytryptamine) is a small lipophile molecule with functional activity taking place in unicellular organisms, plants, fungi and animals. In most vertebrates, including humans, melatonin is synthesized primarily in the pineal gland, On the other hand, melatonin is also produced in other body tissues, including the retina, bone marrow cells, platelets, the gastrointestinal tract, skin and lymphocytes. Melatonin pineal secretion is controlled by the environmental light/dark cycle through the supra charismatic nucleus in the hypothalamus with maximum baseline levels at night [1]. In 1958-1959 by Lerner et al, melatonin has been effectively isolated from bovine pineal extracts and chemically prepared from 5-methoxyindole -3-acetonitrile compound [2].

2.1.2. Melatonin Biosynthesis in the Pineal Gland

The pineal gland regarded as an unpaired neuroendocrine organ located in the midline of the brain. Its primary function is involved in transduction of light and dark information to whole body physiology via the release of hormone melatonin [3]. Beside to melatonin other indoles identified in the pineal gland including serotonin, N-acetylserotonin, 5-hydroxy indole, 3-acetoacetic acid and 5-methoxy indole acetic acid. The enzymatic machinery for the biosynthesis of melatonin in pinealocytes was first revealed by Axelrod [4]. Melatonin is biosynthesized from amino acid tryptophan , which is taken up from the blood into cells by active transport, and converted, via tryptophan hydroxylase and 5-hydroxytryptophan decarboxylase into serotonin, this step is assumed to be the rate limiting in serotonin biosynthesis, Serotonin is then acetylated to form N-acetylserotonin by arylakylamine N-acetyltransferase (AA-NAT), which, represents the rate limiting step in melatonin biosynthesis. N-acetylserotonin is converted into melatonin by hydroxyindole -O-methyltransferase (Fig. 1) [5]. Pineal melatonin production reveals a circadian rhythm, with a low level during daytime and high levels during night. Unlike N-acetylserotonin (NAS) and melatonin that are barely detectable during the daytime, both 5-hydroxytryptophan (5-HTP) and serotonin are secreted at relatively high levels during the day and increase further at night [6].

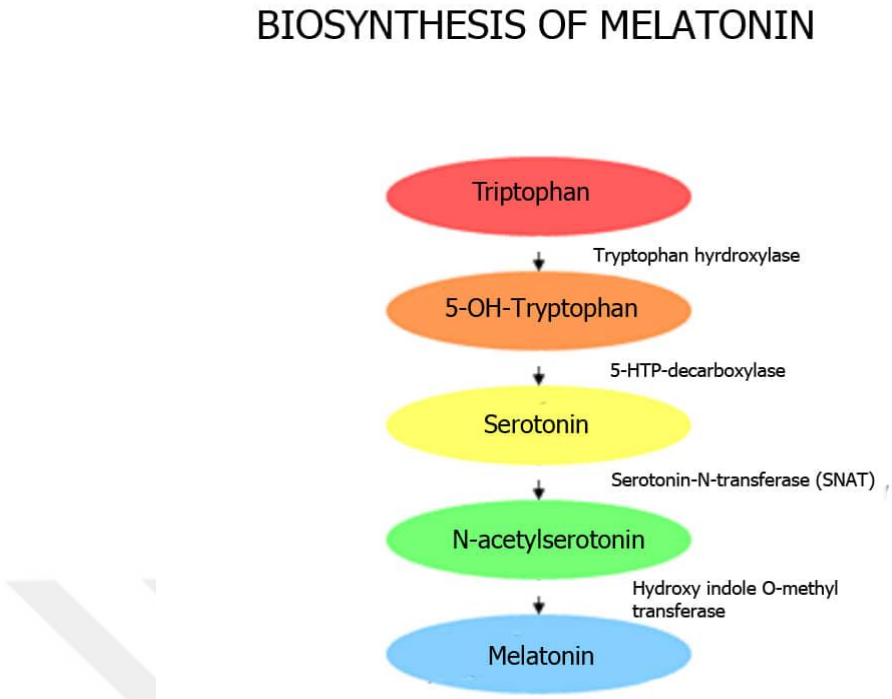

Figure 2. 1. The illustration of melatonin biosynthesis.

2.1.3. Melatonin Biosynthesis in the Pineal Gland Regulation

Melatonin synthesis exhibits a circadian rhythm that is generated by a circadian clock situated in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The SCN clock is set to a 24-hour day by the natural light-dark cycle. The pineal gland is linked to the central rhythm generator in the suprachiasmatic nucleus (SCN) of the hypothalamus via a multi-synaptic pathway [7]. In the absence of the light at night the pineal gland is stimulated by electrical signals arising from suprachaismatic nuclei (SCN) in the hypothalamus, the (SCN) neurons receive inputs from the retina through monosynaptic pathway and send glutamatergic outputs to the autonomic paraventricular (PVN) neurons and then intermediolateral nucleus (IML) of the spinal cord to the the superior cervical ganglion (SCG), fibers from SCG terminate adjacent to the pinealocytes [8]. The neurotransmitter at postganglionic terminal is norepinephrine (NE), its precursor tyrosine, with conversion catalyzed by tryptophan hydroxylase (TH). NE binds to alpha 1 and beta adrenergic receptors on the pinealocyte, the predominant receptors for NE is the beta receptors with highest density at night, NE also binds to alpha receptors for maximal response. The activation of beta receptors stimulates guanine nucleotide binding protein (G-protein), which resulting in separation of G-proteins subunits, which in turn activate adenylate cyclase, ATP is hydrolyzed and intracellular cAMP

rise, which enhances a cAMP- dependent protein kinase, this triggers mRNA expression for the synthesis of NAT enzyme and melatonin. Stimulation of alpha receptors is possible to activate a G-protein –linked phosphatidyl cascade including phospholipase C (PLc), diacylglycerol (DG), and protein kinase C (PKc), which activate adenylate cyclase resulting in increasing NAT activity and melatonin synthesis [9]. During the daytime, a synaptic input from GABAergic SCN terminals to autonomic PVN neurons allows the SCN to block the glutamatergic inputs from PVN. The resulting impact on the preganglionic sympathetic neurons in the intermediolateral nucleus (IML) suppresses melatonin production during the day [10].

2.1.4. Melatonin Biosynthesis in the Extrapineal Tissues

Although the pineal gland is the main site of melatonin synthesis and secretion, the hormone is also found in various tissues of the human body, these include retina, bone marrow cells, platelets, and gastrointestinal tract [10]. The biosynthesis of melatonin in the eye displays the same circadian periodicity. Among other extrapineal sites of melatonin biosynthesis, the gastrointestinal tract is of particular importance as it contains high concentration of melatonin exceeding by hundreds fold those found in the pineal gland. In case of high dietary level of tryptophan, GI melatonin can be released directly into the circulation [11]. The melatonin biosynthesis enzymes have recently been identified in human lymphocytes, and the melatonin that synthesized locally is probably involved in the regulation of the immune system [12].

2.1.5. Melatonin Secretion and Metabolism

Another distinctive feature of melatonin secretion involves that its profile linked to age in humans. Melatonin serum level during first weeks of post-natal life is very low, with no diurnal disparity. At around the sixth month of life the typical diurnal rhythm of its secretion appears, reaching maximum nocturnal levels between the third and sixth years of life. In the course of sexual maturation, a marked decrease in melatonin secretion is noted. At the age of 40–50 years, a marked decrease in daily melatonin synthesis is noted and after the 70th year of age the typical diurnal rhythm of its secretion practically vanishes [13]. In blood melatonin Peak levels vary between individuals and depend on age. High and young secretors have plasma levels of melatonin ranging from 54 to 75 pg/mL, whereas low and elderly secretors range from 18 to 40 pg/mL. The endogenous half-life of melatonin in the serum is 30 to 60 minutes and exogenous melatonin has an even shorter half-life of 12 to 48 minutes, as it is

not stored to any extent. Melatonin has a high lipid/water solubility (octanol/water coefficient = 13) facilitating passage across cell membranes. In the blood, 50% to 75% of melatonin is bound reversibly to albumin and glycoproteins. on other hand, In healthy subjects. melatonin is absorbed from the GI tract and transported by the portal vein system to the liver, where it is metabolized mainly by liver (cytochrome P450 enzyme CYP1A2) to 6-sulfatoxymelatonin (aMT6s). The 6-sulfatoxymelatonin is excreted in urine, and 24-h urine collection of 6-sulfatoxymelatonin is considered as a good index of pineal and extra-pineal melatonin synthesis. An alternative catabolic pathway in the liver includes melatonin oxidation by indoleamine 2,3-dioxygenase and myeloperoxidase to *N*-acetyl-formylo-5-metoxykynuramine (AFMK) and *N*-acetyl-5-metoxykykuramine (AMK). Melatonin is also metabolized non-enzymatically by free radicals and other oxidants. After neutralizing two hydroxyl radicals (OH·), it is converted to cyclic 3-hydroxymalatonin, or it is metabolized to kynuramine derivatives. In some extrahepatic tissues, the process is more intensive, because at low activity of P450 monooxygenase, only a small amount of aMT6s is generated, and AFMK becomes the main product of melatonin oxidation. The extrahepatic melatonin processes and their rates are still poorly recognized. In addition to these, direct interactions of melatonin with reactive oxygen species may increase the activity of antioxidants and antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase (GSH), and induce the downregulation of pro-oxidant enzymes such as nitric oxide synthase and lipogenase [13-16].

2.1.6. Mechanism of Action of Melatonin

Melatonin receptors are found in the body tissues and many cell types, which clarifies its multiple functions in biological rhythms resynchronization, sleep induction, vaso regulation, and even immunomodulation. Several forms of high-affinity (MT1) and low-affinity melatonin receptors (MT2, MT3) have been identified [17]. Each of them contains approximately 350 amino acids and represents around 60% similarity to the other types. This group of receptors belongs to the superfamily of membranous receptors linked to regulatory proteins which bind the G proteins (18), The high-affinity ML1 receptors are designated Mel1a and Mel1b. The Mel1a receptor is expressed in the SCN and in the hypophyseal pars tuberalis. The SCN is the putative site of circadian action of melatonin, and the hypophyseal pars tuberalis is the putative site of its reproductive effects. The Mel1b receptor is expressed mainly in the retina, The ML3 melatonin receptor family is the X-linked orphan G protein-coupled receptor 50 (GPR50) [17], The membranous localization of MT1 and MT2 permits

receiving and transmitting signals across the cell membrane to the interior of the cell [1]. Both MT1 and MT2 play an important role in adenyl cyclase and cyclic AMP (cAMP) inhibition. A decrease in cAMP production reduces the uptake of linoleic acid. Linoleic acid, oxidized to 13-Hydroxy octadecadienoic acid (13-HODE) by 15-lipoxygenase, acts as an energy source for tumor signaling molecules and tumor growth. Inhibition of lineoleic acid uptake by MTN is regarded as a mechanism of its anti-proliferative effects [18]. While ML3 has been subsequently characterized as the enzyme quinone reductase 2 (QR2), a known detoxifying enzyme. Quinone reductases contribute in the protection against oxidative stress by inhibiting electron transfer reactions of quinones, the induction of this enzyme is linked with a decreased susceptibility to cancer initiation and progression. Furthermore, melatonin may act via nuclear receptors belonging to retinoic acid receptor family, RZRα, RORα, RORα2, and RZRβ, with RZRβ being found in neuronal tissues including pineal gland and RZRα in adipose tissue, the skin, testes, cartilage, and liver, RORα, RORα2 appear to be involved in some aspects of immune modulation [19]. Upon binding with nuclear receptors, melatonin modifies the transcription of several genes that participate in cellular proliferation [e.g., 5-lipoxygenase (5-LOX), p21, and bone sialoprotein (BSP) [20].

2.1.7. Antioxidant Activity of Melatonin

The normal cellular metabolism in organisms is totally dependent on oxygen in the process of energy production. A small portion of oxygen (around 5%) undergoes alterations which are disapproving to the body. These alterations result in the formation of reactive oxygen species (ROS) which may damage all cellular components. nucleic acids, lipids, and proteins lead to their oxidation, with subsequent damage, which may be accompanied by further unfavorable alterations in DNA. ROS are believed to participate in a number of cellular/molecular signaling mechanisms that regulate cell growth, differentiation and apoptosis [21]. ROS can serve as growth factor in cancer cells, increased intracellular concentration of these molecular species can result in cancer initiation or growth promotion of previously initiated cancer cells [22, 29]. The most hazardous ROS are the free oxygen radicals superoxide anionic radical (O2–), hydrogen superoxide (H2O2), and the hydroxy radical (OH), which is principally toxic. Free radicals also play an important role in the pathogeneses of several diseases, including cardiovascular diseases, and degenerative diseases of the nervous system [23]. Protective systems both in humans and other aerobic organisms are active against the effects of free radicals. Generally, they can be divided into enzymatic and non-enzymatic mechanisms. The enzymatic system include superoxide

dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT). Endogenous glutathione (GSH), it is not an enzyme, but also can be involved in this category. Non-enzymatic scavengers of oxygen free radicals include, among others, tocopherol (vitamin E), ascorbic acid (vitamin C), b-carotene (provitamin A), coenzyme Q, homocysteine, and many flavonoids. Melatonin at both physiological and pharmacological concentration is one of the most effective substances which protect cells from the action of reactive oxygen species [24-26]. Melatonin's derivatives are also potent free-radical scavengers [27]. Indirectly, melatonin may apply its anti-free-radical action by up-regulating the gene expressions of antioxidative enzymes (SOD, GPx, CAT) [28].

2.1.8. Melatonin Immunostimulatory Function

The relationships between melatonin and the immune system are established by the synthesis of melatonin in bone marrow, in addition, melatonin has been localized in thymus and cells such as mast, natural killer, eosinophilic leukocytes, platelets, and endothelial cells [30]. Melatonin provoke an immunostimulatory effect by its action on T-helper (Th) lymphocytes, although the process may also include mechanisms associated with the effects of melatonin on lymphocyte precursors and natural killer cells [31]. Melatonin acts mostly on the immune system by regulating cytokine production, Melatonin activates T helper cells via increasing IL-2 production, also increases the production of IL-1, IL-6, tumor necrosis factor (TNF)-α, and IL-12 from human and cultured monocytes, IL-2 and IL-12 from macrophage. Melatonin enhances immune function by directly stimulating polymorphonuclear cells, macrophages, NK cells, and lymphocytes [32]. Melatonin is also synthesized in lymphoid tissue, that plays a significant role in the regulation of the human immune system by acting in an autocrine and paracrine manner [30]. Lately, attention has been directed to the effect of melatonin on CD4+ cells. These cells secrete IFN-γ and TNF-α that activate and regulate the cytotoxic T cell response. The Th1 cells directly kill tumor cells by producing cytokines which activate "apoptotic receptors" on the tumor cell surface. NK cells regarded as effective cells against a variety of tumors, particularly leukemias and lymphomas, melatonin also regulates NK activity and improves its cytotoxic effect [33]. These results suggest that melatonin significantly participates in the promotion of the immune system interaction with neoplastic cells.

1.1.9. Melatonin's Effect on Angiogenesis in Cancer

Angiogenesis is a critical step in the development of primary tumors. Angiogenesis is controlled by pro-angiogenic and anti-angiogenic factors in the body. The angiogenesis is regulated by several growth factors such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), Angiogenin. In vitro, pharmacological doses of melatonin decreases serum (VEGF) levels resulting in tumor angiogenesis suppression [34].

1.1.10. Anti-Proliferative Effect of Melatonin

Calmodulin plays an signifcant role in the proliferation of normal and neoplastic cells, calcium activated calmodulin is involved in the initiation of the S- phase of the cell cycle, initiation and completion of M-phase of the cell cycle, and cell cycle –related gene expression. Mitosis, re-entry of quiescent cells from G0 into cell cycle, and rate of cell cycle progression are affected by calmodulin. This progression involves enlargement and de-polymerization of microtubules. The anti-proliferative effect of physiological concentration of melatonin in certain neoplastic cells probably results from an increased degradation of CaM by melatonin /CaM binding and the resulting effect on the CaM concentration distribution between intracellular and extracellular componnets. Ca+2/ CaM stimulate microtubule depolymerization via phosphorylation of tubulin and microtubulin associated proteins (MAPs) via CaM –dependent protein kinase [35], melatonin –calmodulin binding blocks MAPs/CaM and tubulin/ CaM complex formation resulting in cell arrest. Protein kinase C is a major intracellular receptor of diacylglycerol (DAG), the second messenger that play a role in phospholipid metabolism and signaling, several PKC iso-enzymes are Ca+2 –dependent and long term regulation of PKC isoenzymes involved in the regulation of tumor growth, MAPs proteins are phosphorylated by PKC resulting in induction of microtubular alterations required for mitotic spindle and aster formation. physiological and pharmacological concentration of melatonin down-regulate PKC which may play a significant role in melatonin oncostatic action in neoplastic cells [36].

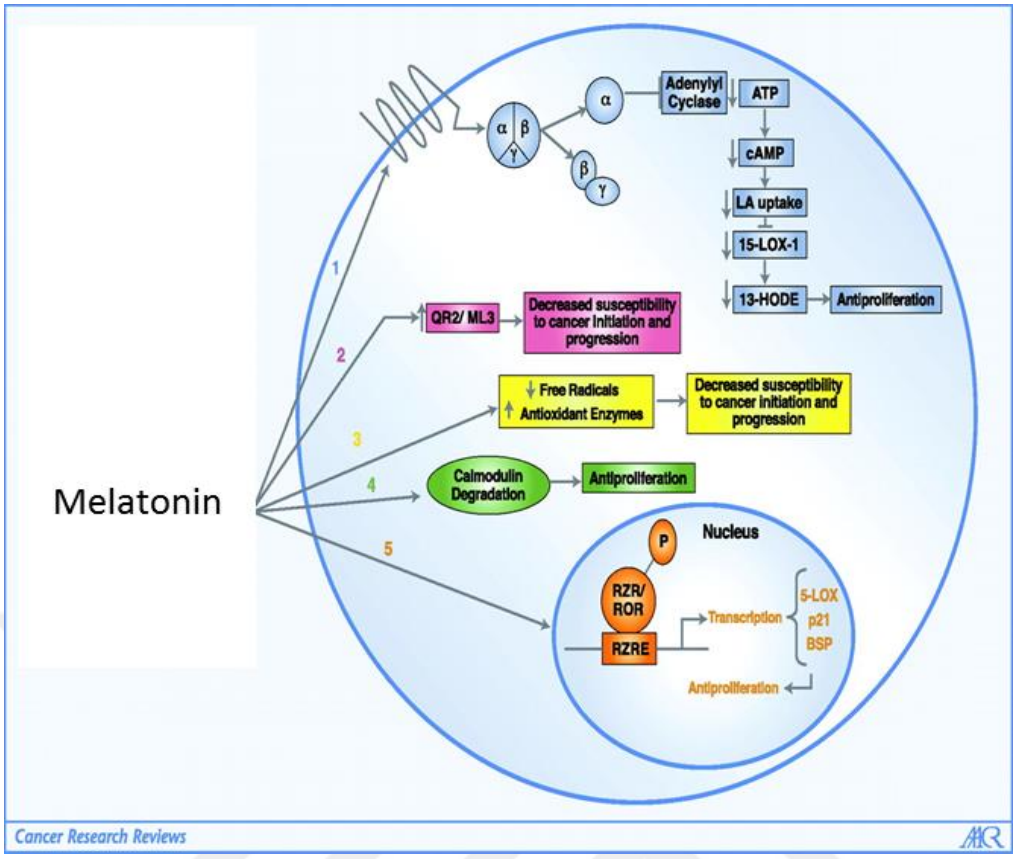

Figure 2.2. Antiproliferative effects of melatonin. These mechanisms [1-5] are shown with different color schemes. 1, (blue), melatonin binds to its receptor(s), ML1 and ML2, the α-subunit dissociates, inhibiting adenylyl cyclase followed by inhibition of ATP and cAMP that leads to a decrease in linoelic acid (LA) uptake followed by decreases in its metabolism to 13-HODE by 15-LOX-1; 2, (red), melatonin can also bind its ML3 receptor, recently reported to be QR2. QR2 is a detoxifying enzyme, which, on induction, decreases the susceptibility to cancer initiation and progression; 3, (yellow), melatonin has been reported to be a powerful scavenger of ROS as well as an inducer of many antioxidant enzymes. Both of these functions lead to a decrease in the susceptibility to cancer initiation and progression; 4, (green), melatonin has been shown to increase calmodulin degradation to result in antiproliferative effects; 5, (gold), melatonin also has been reported to bind to its nuclear receptors, RZR/RORα and RZRβ, altering the transcription of several genes that play a role in cellular proliferation, such as 5-LOX, p21, and BSP, all leading toward anti-proliferation [18].

2.1.11. Melatonin and Apoptotic Pathway

Besides the ability of melatonin to induce cell cycle arrest, it can trigger apoptosis in some cell types and cancer cells, by increasing P53 gene expression [37]. Physiological melatonin with retinoic acid has been described to induce apoptosis in breast cancer cells, but no alterations in the expression of either bax (apoptosis activator gene), bcl-2 (apoptosis suppressor gene) in response to melatonin alone in same cancer cells [38]. In contrast, pharmacological doses of melatonin in vivo applies biphasic effect on apoptosis in colorectal cancer during colon carcinogenesis by inhibiting the cancer during early stages of the disease, whereas stimulating it during the later stages [39]. Therefore, in certain types of tumors under certain circumstances melatonin induced apoptotic cell death may participate in cancer cell growth inhibition.

2.1.12. The Application of Melatonin in Tumor Therapy

Melatonin provokes oncostatic activity through various biological mechanisms involving receptor-dependent and receptor-independent mechanisms [40]. The receptor-independent mechanisms are related to stimulation of anticancer immunity antiproliferative actions, modulation of oncogen expression, and anti-inflammatory, antioxidant and antiangiogenic effects. Additionally, MTN inhibits human cancer cell growth in culture and recent clinical studies appear to confirm its anticancer properties in vivo [18]. MTN have been evaluated in various cell culture lines and experimental animal models, on the other hand, a marked variability among the results of these studies has been possibly detected, because of methodological differences, including study parameters, culture conditions, and different MTN concentrations, doses, and duration of treatment [41]. The timing of melatonin administration appears to be a very important factor in its chemo-preventative properties, with the most effective protocol being a diurnal cycle similar to the physiologic rhythm of melatonin secretion [42]. Studies have proposed that tumor suppressing effects of melatonin tends to be highly effective in the late afternoon, when given to tumor-bearing animals, compared to morning melatonin administration, suggesting that tumors displays their own circadian rhythm of sensitivity [43]. Therefore, it appears that night-time administration of melatonin may be more beneficial than administration during the day time. Among cancer patients, including patients with different types of tumors resistant to standard treatment and/or patients who rejected continuation of the conventional therapy, melatonin is often prescribed as an alternative or complimentary approach because it is thought to be safe as no

adverse side effects have been detected after oral administration of recommended doses [43]. The most possible side effect is the tendency to produce sedation or drowsiness [44]. Various similar studies implemented on patients with advanced metastatic tumors (of the lungs, breast, alimentary tract, head, and neck) received either chemotherapy alone, or chemotherapy plus melatonin. Both one-year survival and regression of tumors confirmed to be considerably more numerous in the patients administered with cytostatic drug plus melatonin. Furthermore, supplementation with melatonin was found to relieve the side effects of chemotherapy (thrombocytopenia, neurotoxicity, cardiotoxicity) [45]. In hormone-dependent tumors, the principal role in growth inhibition tends to be played by the receptor mechanism, which is relatively well documented in breast carcinoma. Melatonin, through its receptors (MT1 and MT2), inhibits the expression of ERa and destabilization of estradiol-ERa complexes and inhibit its binding to ERE (a DNA fragment termed the "estrogen response element"), which prevent the transcription processes in the cell [46]. Additionally, in recently published in vitro studies, melatonin was demonstrated to inhibit testosterone aromatization to estradiol and to serve as an anti-estrogen, blocking ER receptors. Thus, melatonin may be effectively applied as an additional anti-estrogenic factor and aromatization inhibitor in the course of the steroid cycle [47].

2.2. Colorectal Cancer

Colorectal cancer (CRC) is a major health problem throughout the world. [48] It is the third most common type of cancer in men and women and denotes the third most common cause of cancer related death in both sexes, with roughly 600,000 deaths each year. It demonstrates a geographic disparity; It is the fourth most common cancer and the second most common cause of cancer death in the USA, whereas the disease is uncommon in Africa [49]. CRC exists in one of three patterns, these are inherited, familial, and sporadic. The prevalence of colon cancer is assumed to be due to multitude of factors, mainly diet and genetics. Despite the fact that most cases of CRC are thought to be sporadic, which explained by interaction between environmental factors and somatic gene mutation [50], hereditary factors play a significant role, Inherited CRC accounts for 25% of cases, Despite this, only 5% to 10% of CRC cases can be due to mutations in known CRC susceptibility genes, including non–polyposis-associated syndromes mismatch repair genes [MMR] in Lynch syndrome [LS], or polyposis-associated syndromes mostly resulting from adenomatous polyposis coli (APC) gene mutations causing familial adenomatous polyposis (FAP) [51]. further colorectal cancer risk factors possibly include age over 50, inflammatory bowel disease, low fiber and high fat

diet, hypertriglyceridemia, lack of physical activity, obesity, type II diabetes mellitus, alcohol, smoking and others [52-57]. Prognosis of colorectal cancer depends on staging, for localized cancer to the mucosa (stage I and II), Surgical resection for patients results in a 90% 5-year survival rate, but absent from lymph nodes or distant sites involvement [39]. Around 10% of colorectal cancer with distant metastasis still has a poor 5-year survival regardless of aggressive chemotherapy regimen [43]. Furthermore, around 25-50% of colorectal cancer patients whom diagnosed initially with non-metastatic disease experience tumor recurrence regardless of potentially curative resections. In order to improve the prognoses of these patients, it is vital to investigate the etiology and pathophysiology of colorectal cancer and find a novel therapeutic strategy [58-62].

2.2.1. Colorectal Carcinogenesis

Three major pathways for sporadic and inherited colorectal carcinogenesis has been identified; chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP) pathways.

2.2.1.1. Chromosomal Instability Pathway

Most cases of colorectal cancer arise through the CIN pathway.

2.2.1.2. The Adenoma–Carcinoma Sequence

The Adenoma–Carcinoma Sequence, a term that explains the stepwise development of carcinoma from normal epithelium due to accumulation of altered specific genes [63]. the first step of colorectal carcinogenesis is believed to be the inactivation of Adenomatous Polyposis Coli (APC) tumor suppressor gene, followed by stimulating mutations of KRAS, sequential malignant transformation is induced by further genetic mutations in the TGF-β, PIK3CA, and TP53 pathways [64].

2.2.1.3. Genomic Instability and Cancer

For carcinogenesis, the estimated rates of baseline mutation for cellular generation are inadequate to account for the multiple mutations that are demanded for cancer development. The mutation rate per nucleotide base pair is approximated to be as low as 10 - 9 per cellular generation. Thus, it has been suggested that cancer cells must have a “mutator phenotype”, that resulting in increasing their rate of spontaneous mutations. In sporadic colorectal cancer around 65%–70% shows Chromosomal instability (CIN), this term refers to an accelerated

rate of gains or losses of whole or large portions of chromosomes that lead to karyotypic variability between the cells. The results of CIN involve an imbalance in chromosomal number (aneuploidy), subchromosomal genomic amplifications, and a high incidence of loss of heterozygosity (LOH) [64].

2.2.1.4. Adenomatous Polyposis Coli (APC) Tumor Suppressor Gene:

Mutation of the adenomatous polyposis coli (APC) tumor suppressor protein is responsible for both inherited and sporadic forms of colon cancer. absolutely how inactivation of APC participate in the tumor progression is unknown. The Apc gene (APC), essentially a cytoplasmic protein, inhibits cell cycle progression and has a critical roles in development. The APC binds to β-catenin, axin and glycogen synthase kinase 3β resulting in a large protein complex formation, in which β-catenin is phosphorylated and broken down, producing negative regulation of the Wnt signaling pathway. The commonest targets for APC genes mutation is the β-catenin-binding regions preventing downregulation of the β-catenin and activation of Wnt signaling pathway, leading to tumor cell overproliferation [65].

2.2.1.5. APC/ β –Catenin/ WNT Signaling Pathway

Approximately 70% of sporadic colorectal cancer and most of the familial adenomatous polyposis (FAP) cancer predisposition syndrome is due to Mutations in the adenomatous polyposis coli or β-catenin gene. That lead to increase cytosolic level of β-catenin and, subsequently, to activate the β-catenin–T cell-factor/lymphoid-enhancer-factor complex transcriptional ability. This process seems to play important role in the colorectal carcinomas development [66]. In normal mucosa, β-catenin Together with α- and γ-catenin play a role in cell–cell adhesion, whereas β-catenin binds to the transmembrane protein E cadherin domain in the cytoplasm to form a complex that is necessary for adhesion to occur, (APC) competes with E cadherin for binding to β-catenin internal repeats, cytoplasmic β -catenin protein is continually removed by the action of the Axin complex, which is formed of the scaffolding protein Axin, (APC) gene product, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3). CK1 and GSK3 subsequently phosphorylate the APC/β-catenin complex resulting in recognition of β-catenin by b-Trcp, an E3 ubiquitin ligase subunit, and successive β-catenin ubiquitination and proteasomal degradation, this continual removal of β-catenin inhibits β-catenin from entering the nucleus, thereby Wnt target genes are repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) proteins. Disruption of the APC protein activate Wnt signaling pathway increasing transcription of WNT target

genes, which has a major role in tumor growth and invasion by its interaction with the T-cell factor/lymphoid enhancer factor family of transcription factors [67].

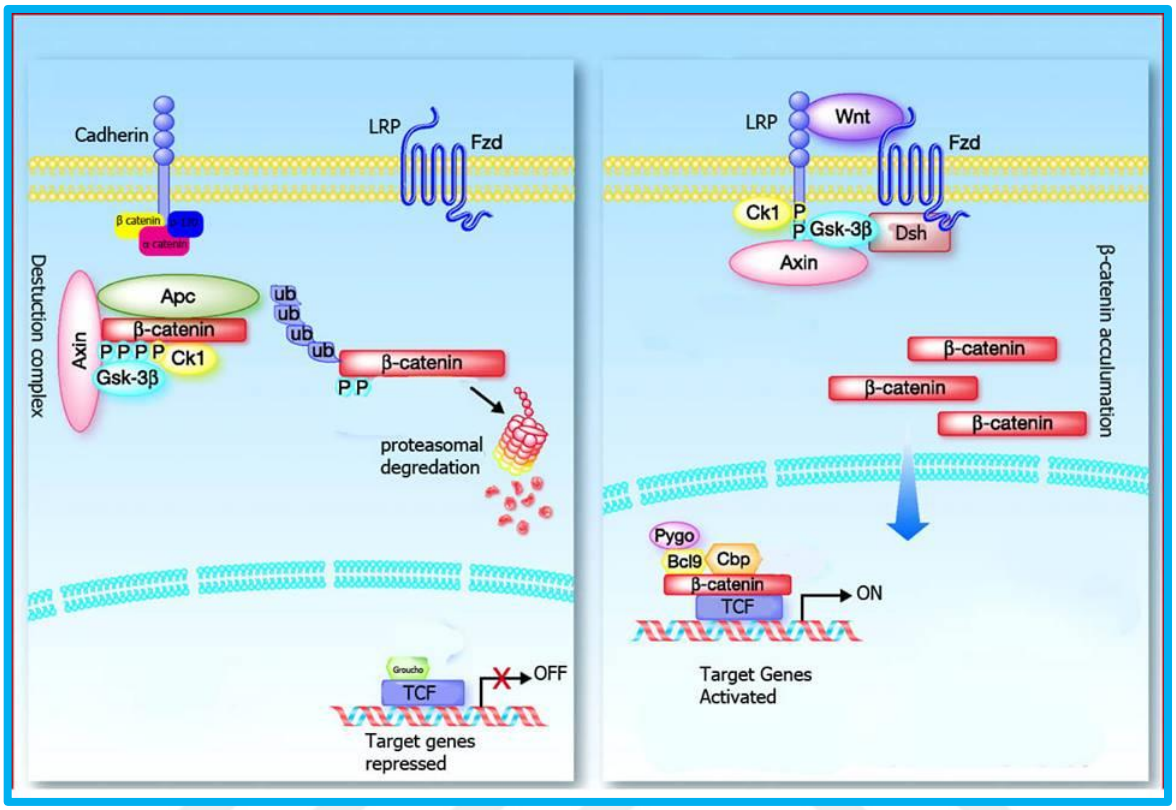

Figure 2.3. The Wnt signaling pathway in the "OFF" and "ON" states. In conditon that the Wnt signal is absent, cytoplasmic β-catenin constantly degraded by the Axin complex, which is formed of the scaffolding protein Axin, (APC) gene product, casein kinase 1 (CK1), and glycogen synthase kinase 3 (GSK3) in a proteasome-dependent manner, on other hand, Wnt target genes are also kept silent and are repressed by the DNA-bound T cell factor/lymphoid enhancer factor (TCF/LEF) proteins. In the presence of a Wnt ligand, it binds to the transmembrane Frizzled (Fz or Fzd) receptor and its coreceptor, low-density lipoprotein receptor-related protein 6 (LRP6), or LRP5 forming Wnt-Fz-LRP6 complex, simultaneously with the recruitment of the scaffolding protein Dishevelled (Dvl), that lead to LRP6 phosphorylation and activation and the recruitment of the Axin complex to the receptors. These consequences inhibit Axin-mediated b-catenin phosphorylation and thus stabilization of β-catenin that accumulates and enters the nucleus to form complexes with TCF/LEF and stimulate Wnt target gene expression [67].

2.2.1.6. K-RAS (Kirsten rat sarcoma gene):

KRAS is a proto-oncogene encoding a small 21 kD guanosine triphosphate/guanosine diphosphate binding protein that has a function in the regulation of cellular response to many different stimuli. Mutation of KRAS genes in form of DNA base pair substitutions within codons 12 and 13 of exon 2, each substitution leads to amino acid substitution of protein, thereby resulting in GTPase inactivity and stimulation of RAS/RAF signaling pathway that are found in 35% to 42% of CRCs particularly in early carcinogenesis. Seven different DNA base pair substitutions within codons 12 and 13 of exon 2, each leading to an amino acid substitution in the protein [68]. RAS signaling activation regulates many cellular functions by activation of the Raf–mitogen-activated protein kinase (MEK)–extracellular signal-regulated kinase (ERK) pathway, The Raf activate MEK1 and MEK2, that subsequently phosphorylate ERK1 and ERK2 that in turn phosphorylates cytosolic and nuclear substrates. These substrates regulate enzymes which have a role in the control of cell cycle progression [69]. Despite MEK is usually activated in colorectal cancer, recent studies revealed that inhibitors of MEK are ineffective therapeutics [70]. RAS–guanosine triphosphate moreover binds the catalytic subunit of type I phosphatidylinositol 3 kinases forming phosphoinositol lipids. PIK3CA is mutated in almost 20% of CRCs [71].

2.2.1.7. TP53

The human TP53 gene is positioned on the short arm of chromosome 17p, and is formed of 11 exons and 10 introns. p53 protein (whild type) consists of 393 amino acid residues, and numerous functional domains, p53 considered to be a chief regulator for hundreds of genes involved in DNA metabolism, apoptosis, cell cycle regulation, angiogenesis, immune response, cell differentiation, motility, and migration. Tp53 is the most frequently mutated gene in differnent types of tumor, p53 mutations considered to have critical role in malignant transformation from adenoma to carcinom through pathological process of the tumor. approximately 34% of the proximal colon tumors, and 45% of the distal colorectal tumors have p53 mutation, Majority of these mutations occur in exon 5 to 8 (DNA binding doman), and mainly missense mutations in codons, such as 175, 245,248, 273 and 282, causing DNA binding disruption [71, 72].

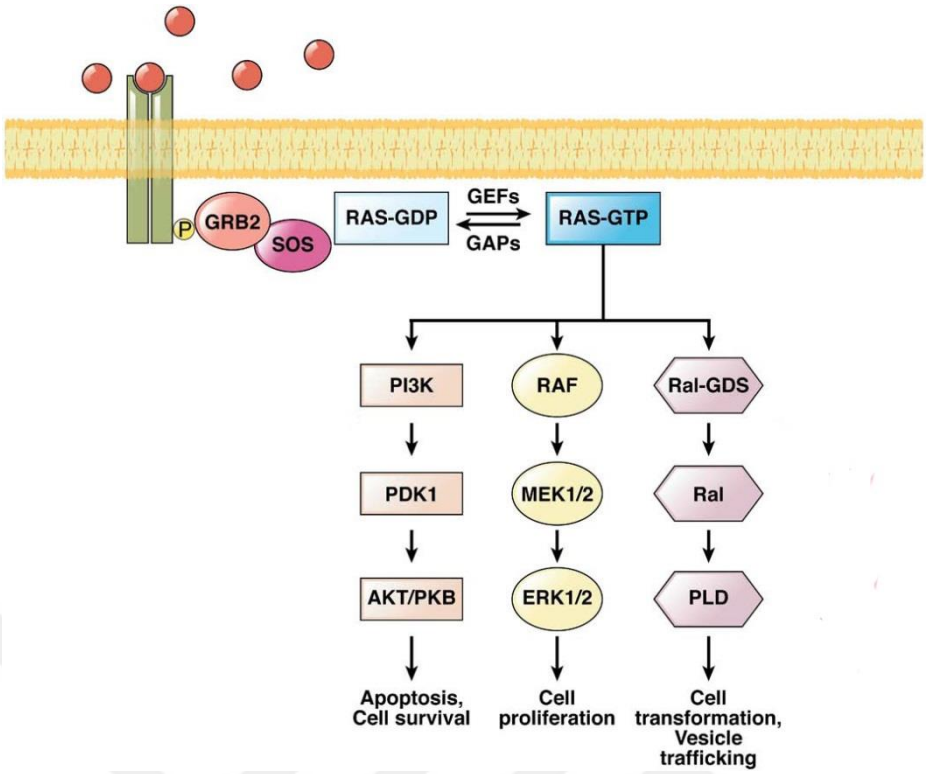

Figure 2.4. The RAS signaling pathway. RAS activation occurs as a result of binding of growth factors to cellular receptos that stimulate guanine exchange factors (GEF), such as SOS (son of sevenless) that linked to the adaptor protein GRB2 (growth-factor-receptor bound protein 2). SOS activate the release of bound guanosine diphosphate (GDP) from RAS, and it is replaced by guanosine triphosphate (GTP), producing the active RAS-GTP conformation. The guanosine triphosphatase (GTPase)-activating proteins (GAP) binds to RAS-GTP increasing the conversion of RAS-GTP to RAS-GDP, thus eliminates signaling. Mutated RAS is basically active in the RAS-GTP conformation. Activated RAS controls multiple cellular functions through effectors involving the Raf–MEK–ERK pathway, phosphatidylinositol 3 kinase (PI3K) [71].

2.2.1.8. Aneuploidy 18q Loss:

Allelic loss at chromosome 18q is identified in ~70% of primary CRC mainly in the late stages of the disease, and could represent a negative prognostic marker for survival in patients with CRC. The high tendency of allelic deletions including chromosome 18q proposes the existence of candidate tumor-suppressor genes whose inactivation may have an important role in CRC, including DCC, SMAD2 and SMAD4 [64].

2.2.2. Microsatellite Instability in Colorectal Cancer:

In 1992, by Manuel Perucho [73] thousands of DNA sequences have been extracted from colonic cancer tissues and compared with adjacent normal colonic tissue and then amplified using a polymerase chain reaction technique (PCR). Careful analysis proposed that tumors had bands that were actually shorter in length rather than deleted, the analysis of band sequences discovered simple repetitive sequences (ie, microsatellites), primarily in polyadenine (An) tracts associated with Alu sequences. MSI is demonstrated in approximately 12 of all colorectal cancers. The tumors with these somatic mutations had characteristic clinical and pathological features, including a tendency to arise in the proximal colon, were less invasive, were estimated to have less KRAS or p53 mutations, in which the tumor were poorly differentiated. The colorectal cancer with MSI mutations have to some extent a better prognosis than those without MSI mutations and have a different response to chemotherapy [73].

2.2.3. Role of the Serrated Pathway in Colorectal Cancer Pathogenesis:

2.2.3.1. Histological Classification of Serrated Colorectal Polyps:

Hyperplastic polyposis syndrome (HPS) is a relatively considered as rare condition that exhibits a numerous hyperplastic polyps found in a pancolonic distribution; some of these polyps are approximately 1 cm in diameter [66].

Histological Classification	***Abbreviation***	***Features Of Polyp Type***
Hyperplastic polyps goblet cell –rich type microvesicular type	GCHP MVHP	Typically small polyps in the distal bowel with normal architecture and proliferation , both types in the upper crypt.
Sessile serrated adenoma	SSA	Distinction from hyperplastic polyps based mainly on abnormal architectural features including branching of the crypts, dilation of the base of the crypts, and abnormal proliferation

Traditional serrated adenoma	SA or TSA	Does not contain sessile serrated adenomas, these polyps are defined by an overall protuberant growth projections with dysplastic cells containing elongated nuclei and eosionophilic cytoplasm.
Mixed serrated polyp	MP	Individual components should be listed

Table 1. The classification of serrated colorectal polyps [66].

2.2.3.2. Molecular Pathogenesis of the Serrated Pathway—The Central Role of CIMP and BRAF

2.2.3.2.1. The Central Role of CIMP in the Neoplastic Serrated Pathway

Another molecular pathogenic pathway has been described in CRC, that is CpG island (CGI) methylation. DNA can be methylated by adding a methyl group to a cytosine base. As the DNA sequence is not changed, this is known as an epigenetic rather than genetic alteration. The combination of a cytosine nucleotide successively followed by a guanine nucleotide (CpG dinucleotide) resulting in formation of dense clusters of CpG dinucleotides, termed CpG islands that found in the promoter regions of almost 50% of all genes. Aberrant hypermethylation of these promoter CpG islands has a central role in tumor growth. Regarding methylation, two types were recognized: type A methylation, which has been associated with old age group, and type C that particularly was related to cancers, it is estimated that around 20%–30% of all colorectal cancer have methylated type C genes, this phenomenon was known as CIMP, whereas the majority of sporadic MSI associated tumors have CIMP, it is not restricted to this tumor variety; roughly 50% of all cancers with CIMP have no methylation of MLH1 or MSI [66].

2.2.3.2.2. The Central Role of BRAF in the Neoplastic Serrated Pathway

The oncogene BRAF mutation detection in cancer cells with MSI and serrated polyps was very advantageous to advance our understanding of the serrated neoplastic pathway. A particular, initiating, point mutation in BRAF (V600E) resulting in important signaling of both the mitogen-activated protein kinase (MAPK) pathway and KRAS that leads to cell proliferation, persistence, and inhibition of apoptosis, BRAF mutation was detected in the

most of SSA (Sessile serrated adenoma) but roughly not in conventional adenomas. Moreover, BRAF is not altered in HNPCC (hereditry non-polyposis colorectal cancer), indicating the association with the serrated pathway rather than MSI [66].

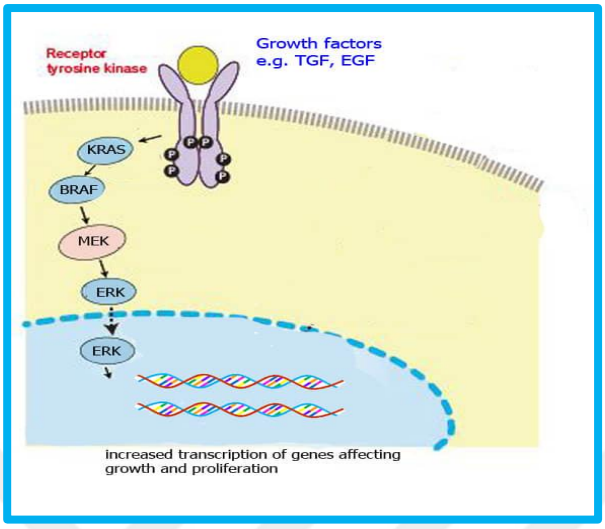

Figure 2.5. KRAS and BRAF mediate transduction of signals from extracellular stimuli via the mitogen-activated protein kinase (MAPK) signaling pathway. Activated extracellular signal-regulated kinase (ERK) can translocate to the nucleus, where it activates transcription factors to alter expression of genes that regulate cell growth and proliferation. Mutations in the oncogenes KRAS or BRAF result in constitutive activation of the pathway and uncontrolled cell proliferation. EGF (epidermal growth factor, TGF (tissue growth factor) [66].

2.3. Melatonin and Colorectal Carcinoma

Several studies have demonstrated reduced levels of melatonin in patients with certain types of cancers, compared with normal, healthy people of the same age (13,68-70) [42, 44, 74, 75]. Many cancer types [breast, non–small cell lung, metastatic renal cell carcinoma (RCC), hepatocellular carcinoma, and brain metastases from solid tumors] have proved to be responsive to melatonin in different settings, and several others (ovarian carcinoma, human neuroblastoma, pituitary tumors, larynx carcinoma, oral carcinoma cells, bladder carcinoma, and erythroleukemia) are under investigation [14, 42]. In several studies, melatonin has revealed anticancer potency for various colorectal cancers. Melatonin's oncostatic effect through receptor-independent mechanisms involving increased ROS levels and decreased cellular viability, antioxidative and anti-inflammatory activities, cell proliferation inhibition, apoptosis induction, at pharmacological concentrations in a dose-dependent manner [76]. The

oncostatic effect mechanisms were described by histone deacetylase 4 (HDAC4) nuclear import and subsequent H3 deacetylation via the inactivation of Ca2+/calmodulin-dependent protein kinase IIα (CaMKII). A study demonstrate that melatonin might suppress tumor growth and progression of colon carcinoma via blocking the activation of ET-1 [77], Endothelin-1 (ET-1) is a peptide, which serves as a protector of carcinoma cells from apoptosis and promoter of angiogenesis [78]. In another study the oncostatic effect of melatonin on colon cancer was proved to be mediated through MT2 receptors and through its binding to nuclear RZR/RORα receptor [79].

2.4. Paclitaxel

In the late 1960s, the paclitaxel was first described and isolated from the Taxus brevifolia Pacific yew tree. Paclitaxel is considered as one of the cytotoxic diterpene compounds that are one of the most effective anticancer agents which are broadly used alone or in combination against solid malignancies, lung, head and neck, ovarian, and breast cancers [80]. Microtubules have a critical role in different processes of eukaryotic cells including cell mitosis, motion, intracellular trafficking and conformational adaptation to interact with the environment, the intracellular functions of microtubules are controlled to great extent by their polymerization dynamics. Polymerization mechanism of microtubules takes place by adding the reversible non-covalent α and β tubulin dimers at both ends resulting in nucleation-elongation. The paclitaxel is acting by stimulating microtubule Polymerization through its binding to the β-tubulin subunit that promotes conformational changes of the M-loop of β-tubulin that leads to more stable lateral interactions among adjacent proto-filaments, subsequently stabilization of polymerized microtubules and prevention of the normal dynamic function of the cytoskeleton that leading ultimately to mitotic arrest and the cells finally die by apoptosis. The common toxicities of paclitaxel are neurotoxicity and myelosuppression (dose-limiting toxicity) and MDR, the effectiveness of paclitaxel for cancer treatment has been reduced, however, by these side effects especially drug resistance. Identifying new strategies to reduce side effects and avoid drug resistance remains a significant challenge [80, 81].

3. MATERIALS and METHODS

3.1. Cell Culture and Reagents

The human colorectal carcinoma cell line HCT-116 was a kind gift from Prof Dilek Telci, Yeditepe University, Turkey. Cells were maintained in DMEM-F12 medium (PAN Biotech, P04-41500) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, 10500) and penicillin (50 µg/mL)/streptomycin (50 µg/mL)/neomycin (100 µg/mL) mixture (Gibco, 15640055) at 37 °C under 5% CO_2 humidified atmosphere in 75 cm^2 tissue culture flasks (SPL Life Sciences, 70075).

Cells were routinely passaged when they reach the 80 % confluency. Basically, cells were washed with 8 – 10 mL Dulbecco's phosphate buffered saline (DPBS, Gibco, 14190-094) and incubated in 1 mL Trypsin-EDTA (0.25%; Gibco, 25200056) for 2 – 3 min until the cells were detached from the flask. Then, 8 – 10 mL medium was added to the flask and cells were resuspended to obtain single cells, transferred to a new flask or cell suspension was prepared for the further experiments.

Cells were counted on a Countess™ II FL Automated Cell Counter (Thermo Fisher Scientific, AMQAF1000) by using Countess™ Cell Counting Chamber Slides (Invitrogen™, C10228). Basically, equal amounts of trypan blue (0.4 %; Invitrogen™, C10228), which is a dye used in the discrimination of the dead and viable cells as the dye enters into cells if the cell is damaged[86] cell suspension were mixed thoroughly by pipetting and 10 µL of mixture was added to each chamber of the slide. Then, the slide was inserted to instrument and number of viable cells per mL was calculated.

Paclitaxel (PTX, Sigma-Aldrich, T7402) and melatonin (MTN, Sigma-Aldrich, M5250) stocks with intended concentrations were prepared in DMSO.

3.2. Cell Viability Assay, Cell Counting and Size Measurements

The water soluble tetrazolium-1 (WST-1) colorimetric proliferation assay (Roche, 5015944001), which is based on the cleavage of the WST-1 salt by the mitochondrial dehydrogenases to formazan in the metabolically active cells, was used to determine the toxicity of PTX and MTN. HCT-116 cells with a density of 5 x 10^3 cells in 100 µL/well were seeded in a 96-well plate. After overnight attachment, cells were treated with PTX or MTN or combination of PTX and MTN for 24-48 h with the indicated concentrations and schemes.

Next, 10 µL WST-1 reagent was added to each well, and plates were shaken for 1 min at 600 rpm, then incubated for an additional 2 h at 37 °C. At the end of the incubation period, plates were read at 440 nm using a Synergy HT microplate reader (BioTek Instruments, Inc., USA). The readings were then normalized against the control group and presented as percent of control.

Cells were counted as previously described and 3 x 10^5 cells were seeded in 6-well plates (Sigma-Aldrich, CLS3516). After overnight attachment, the cells treated with PTX or MTN or combination of PTX and MTN for 24-48 h with the indicated concentrations and schemes. Next the cells were washed with 3 mL DPBS and detached from the surface by incubating with 300 µL Trypsin-EDTA (0.25%; Gibco, 25200056). After detachment, 700 µL medium was added to the wells, and cells were resuspended and collected in 1.5 mL Eppendorf tubes. As the next step, the cells were counted as described previously and the number of the cells and cell diameter were noted.

3.3. Apoptosis and Flow Cytometry Analyses

Cells with a density of 5 x 10^5 cells in 4 mL were seeded on 6 cm dishes. After overnight attachment, cells were treated with PTX (50 nM) or MTN (500 µM) or combination of PTX and MTN for 24-48 h with the indicated schemes in order to analyze the apoptotic activity and protein levels of active Caspase-3, as apoptosis induction marker, and LC3A/B, as autophagy marker. After treatments for indicated times, floating cells were collected in a sterile 15 mL canonical tube and non-floating cells were collected after trypsinization. Cell suspension was divided into two tubes with equal volumes and each of them was washed with 1 mL DPBS (Gibco, 14190-094). For the measurement of the apoptotic activity, 1 mL of the cell suspension was added in the canonical tube containing floating cells, centrifuged at 300 x G and stained with Annexin V-FITC and propidium iodide according to manufacturer's instructions (MEBCYTO® Apoptosis Kit; Cat. No. 4700, MBL International). Then the cells were subjected to flow cytometric measurements in a Navios Flow Cytometer (Beckman Coulter) and the results were analyzed on Kaluza Analysis Software (ver. 1.5a, Beckman Coulter).

In order to analyze the levels of active Caspase-3 and LC-3A/B, the remaining 1 mL of cell suspension was centrifuged and fixed with 1 mL 4 % paraformaldehyde at 4 °C for 45 min, centrifuged at 2000 rpm for 5 min and supernatant was discarded. Cells were permeabilized with 500 µL filter-sterilized permeabilization solution (PERM; 1 % saponin

(Sigma, 47036) and 1 % FBS (Gibco, 10270, (Heat-inactivated at 57 °C for 2 h before use) in 500 µL Isoflow (Beckman Coulter, 8546859)) at 4 °C for 30 min and centrifuged at 300 x G for 5 min. Then the cells were incubated with 5 µL Caspase-3 (BD Biosciences, PE-conjugated, 51-68655X), and 1 µL LC3A/B (Cell Signaling Technologies, #4108) at 4 °C for 20 min in 500 µL PERM and centrifuged at 300 x G for 5 min. Cells were then incubated with 1µL sheep anti-rabbit IgG H&L (DyLight® 488) (Abcam, ab96923) 4 °C for 20 min in 500 µL PERM and centrifuged at 300 x G for 5 min. Cells were then resuspended in fresh PERM and subjected to flow cytometric measurements in a Navios Flow Cytometer (Beckman Coulter). The results were analyzed on Kaluza Analysis Software (ver. 1.5a, Beckman Coulter).

3.4. Statistical Analysis

Statistical analyses were conducted using SPSS 18.0. All results are represented mean ± standard deviation. Statistical analysis methods were indicated in the figure legends, and the p values equal to or lower than 0.05 accepted as statistically significant.

4. RESULTS

4.1. Melatonin treatment has a tendency to decrease the cell viability in a dose- but not time-dependent manner

To analyze the cytotoxic effects and treatment time of MTN, the HCT-116 cells were treated with different doses of MTN for 24 and 48 h. MTN treatment led to a dose-dependent decrease in the cell viability at both 24 and 48 h ($p<0.051$) (**Fig.4.1.**). Reduction tendency in cell viability was observed at 250 and 500 μM MTN at 24 h, and 48 h compared to their respective control groups.

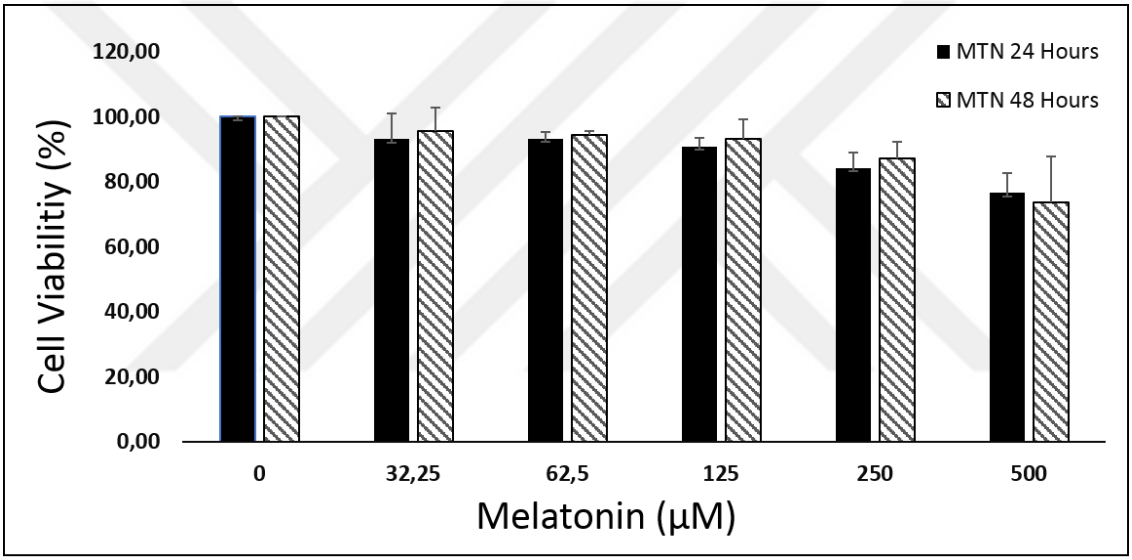

Figure 4.1. The effect of dose and time dependent melatonin treatment to the HCT-116 human colon cell line on the cell viability.

4.2. Paclitaxel treatment decreases the cell viability in a dose- but not time-dependent manner

To analyze the effective dose and treatment time of PTX, the HCT-116 cells were treated with different doses of PTX for 24 and 48 h. PTX treatment caused a dose-dependent reduction in cell viability at 10 and 50 nM PTX at 24 and 48 h h compared to their respective control groups, *p<0.05. (**Fig. 4.2.**).

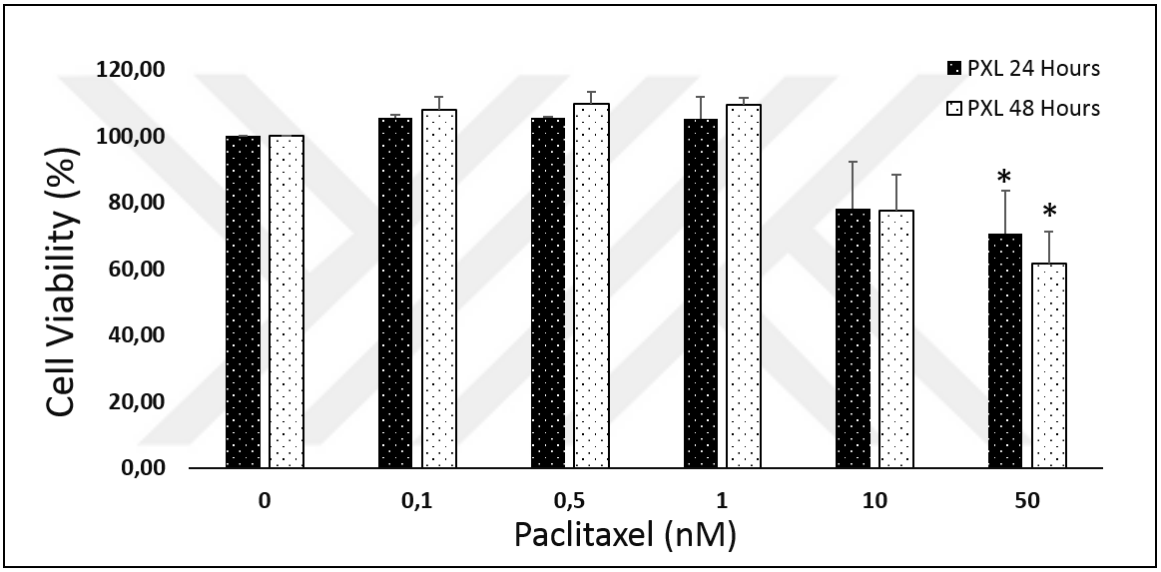

Figure 4. 2. The effect of dose and time dependent paclitaxel treatment to the HCT-116 human colon cell line on the cell viability. * p<0.05 the difference from respective control group)

4.3. Melatonin potentiates the cytotoxic effects of paclitaxel in a dose-dependent manner

The three doses of MTN (125, 250 and 500 μM) significantly reduced the cell viability when combined with PTX (50 nM) compared to MTN alone treated groups at both 24 and 48 h (**Fig. 4.3.**) These results indicate that **MTN increases the cytotoxic effects of PTX in a dose independent manner.** Kruskal Wallis and Mann Whitney U comparison test were used to define the difference between all groups and related two groups respectively.

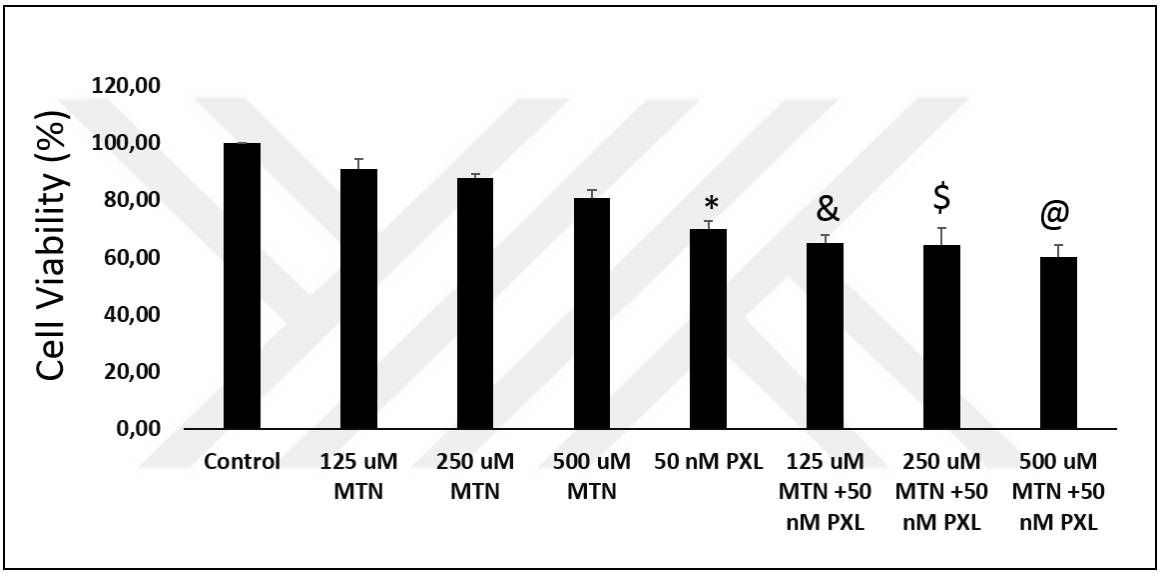

Figure 4.3. The effect of dose dependent melatonin treatment with paclitaxel treatment on the cell viability of HCT-116 human colon cell line for 24 h. Data are mean ± S.D. (n=3 independent experiments).* $p<0.05$ difference from control group, & $p<0.05$ difference from 125 μM MTN treated group, $ $p<0.05$ diference from 250 μM MTN treated group, @ $p<0.05$ difference from 500 μM MTN treated group.

4.4. Apoptosis induction by MTN and PTX differs depending on the treatment scheme

We further investigated that whether the MTN and PTX decreases the cell viability differentially depending on the treatment scheme due to the differential apoptosis induction. Treatment with 500 μM MTN alone for 24 h did not induce either apoptosis. On the other hand, treatment with 50 nM PTX (**p<0.01) and the combination of MTN and PTX (@@ p<0.01) significantly induced the early apoptotic events at 24 h (Kruskal Wallis followed by Mann Whitney U), when compared to 24 h MTN and 24 h MTN and PTX respectively. Interestingly, as shown in figure 4.4. we observed while the HCT-116 cells treated with MTN for 24 h and then treated with PTX next 24 h caused to increase the apoptosis when compared to 24 h MTN alone treated group (@ p<0.05) but did not induce apoptosis when compared to 24 h PTX alone treated group but if the cells treated with PTX for th e first 24 h and then treated with MTN for the next 24 h, caused to increase the apoptosis when compared to 24 h PTX alone treated group (Ø p<0.05)

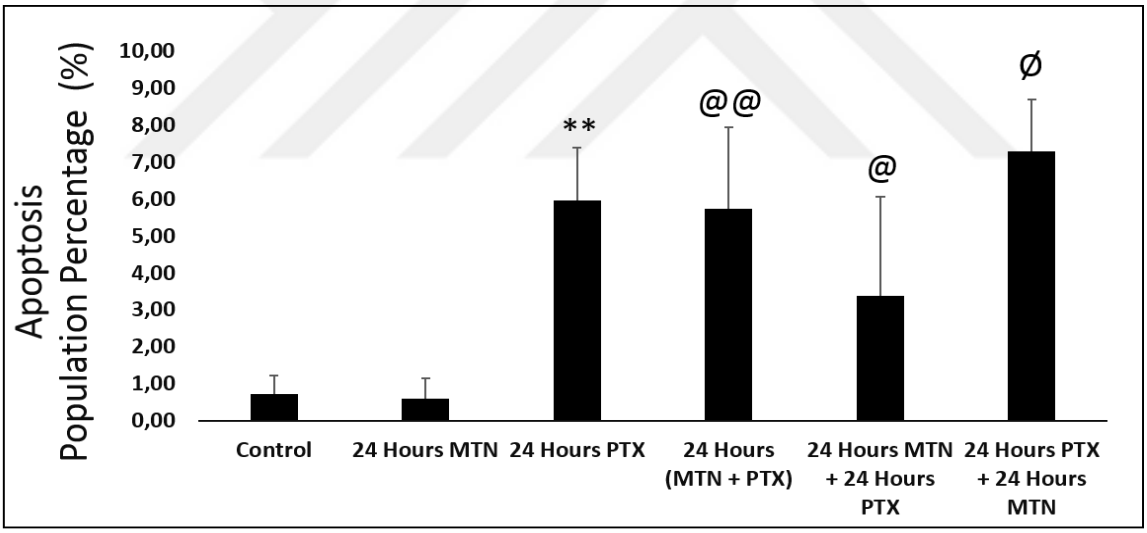

Figure 4. 4. The effect of melatonin and/or paclitaxel treatment to the HCT-116 human colon cell line on the apoptotic population percentage.** p<0.01 difference from control group, @p<0.05 difference from 24 h MTN treated group, @@ p<0.01 difference from 24 h MTN alone treated group. Ø p<0.05 difference from 24 h PTX alone treated group.

4.5. Necrosis induction by MTN and PTX differs depending on the treatment scheme

We investigated that whether the MTN and PTX decreases the cell viability differentially depending on the treatment scheme due to the differential necrosis induction. Treatment with 500 μM MTN alone for 24 h or 50 nM PTX alone or combination of MTN and PTX for 24 h did not induce either the necrosis. On the other hand, if the cells treated with 500 μM MTN for first 24 h and then treated with 50nM PTX for next 24 h caused to increase in necrosis when compared to 24 h PTX alone treated group (@$p<0.05$) and if the cells treated with PTX for th e first 24 h and then treated with MTN next 24 h, caused to increase the necrosis ratio when compared to 24 h PTX alone treated group (Ø Ø $p<0.01$)

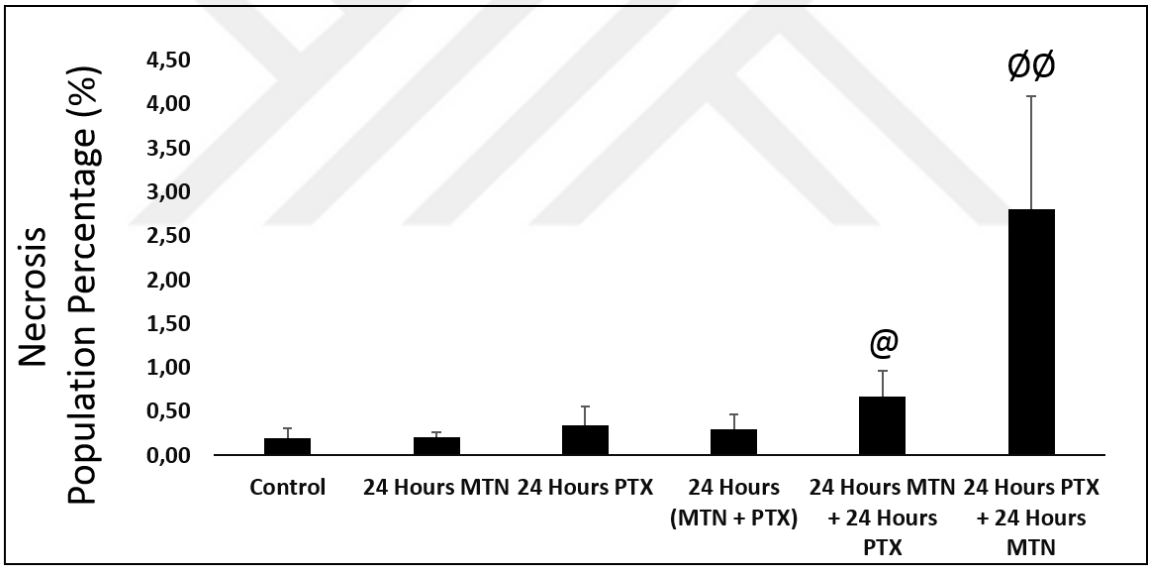

Figure 4. 5. The effect of dose dependent melatonin and/or paclitaxel treatment to the HCT-116 human colon cell line on the necrotic population percentage. @ $p<0.05$ difference from 24 h PTX alone treated group, Ø Ø $p<0.01$difference from 24 h PTX alone treated group.

5. DISCUSSION and CONCLUSION

In this study, the cytotoxic effects of PTX and MTN were investigated on human colon cancer cells *in vitro*. The conventional treatment of CRC is based on the resection and chemotherapy [21]. However, treatment of colon cancer with chemotherapeutics is challenging as the most of the patients acquire resistance to the drugs, leading to decreased response to the treatment [24], the chemotherapeutics are not only toxic to the tumor but also to the health cells [21]. Therefore, combination of the chemotherapeutics with other cytotoxic agents in order to improve the treatment outcome is a possible approach [21].

PTX is a widely used chemotherapeutic drug against ovarian, breast and lung cancer [6] and is stated not to be efficient in the colon cancer treatment [11]. Although there have been mechanisms suggested for the cytotoxic effects of PTX, it is still unknown how PTX kills the cancer cells [6]. Basically, PTX induces microtubule polymerization and stabilization, leading to an arrest of the chromosome segregation at the spindle checkpoint during the cell cycle [6]. This causes unequal distribution of the chromosomes in the daughter cells and consequently cell death [25]. Besides the growth arrest, PTX treatment induces apoptosis by either directly or indirectly affecting mitochondria [26-29]. Additionally, inhibition of mitogen-activated protein kinase (MAPK) pathway that is deregulated in many types of cancer [31] was shown to increase the PTX-induced apoptosis in colon cancer cell lines [30]. Resistance to the microtubule-targeting drugs were also stated to be linked with increased activity of phosphatidylinositol 3-kinase (PI3K)/Akt and MAPK pathway in prostate cancer cells [32]. Another study showed that PTX deregulates the autophagy by decreasing the autophagosome maturation, localization and trafficking, however, autophagy assists the PTX-induced apoptosis in breast cancer cells [82].

Various mechanism for anticarcinogenic effects of MTN was suggested both *in vitro* and *in vivo* [14]. The studies regarding the colon cancer suggested that MTN exerts its activities through regulation of reactive oxygen species (ROS) levels [63], promotion of cell cycle arrest at G_1 phase [16], modulation of autophagic and Nrf2 pathways [48], apoptosis induction [49] and downregulation of migration through p38/MAPK pathway [51]. Therefore, combinatorial treatment is now of interest. Besides the present study showed that PTX and MTN alone or in combination showed a dose dependent cytotoxicity. Further, cytotoxicity differed and were improved depending on the treatment scheme.

Further investigations we need to perform more studies and experiments to explain the related mechanisms by molecular level.

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7. CURRICULUM VITAE

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Degree	**Department**	**The name of the Institution Graduated From**	**Graduation year**
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Turkish	Advanced

Work Experience (Sort from present to past)

Position	**Institute**	**Duration (Year - Year)**
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Computer Skills

Program	**Level**
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Microsoft Power Point	Advance