FACULTAD DE MEDICINA

UNIVERSIDAD DE CANTANBRIA

GRADO EN MEDICINA

TRABAJO FIN DE GRADO

Melatonin, chemotherapy, and altered gene expression in cancer

Melatonina, quimioterapia, y expresión génica alterada en el cáncer

Autor: Teresa Díez Alonso

Director/es: D. Carlos M. Martínez-Campa

Santander, junio 2017

Abstract

Melatonin is a hormone synthesized and released by the pineal gland following a circadian rhythm, with low levels during the day and elevated levels at night. This indoleamine mitigates cancer at the initiation, progression and metastasis phases. Some of the anticancer actions depend of two melatonin high-affinity G protein-coupled receptors, termed MT1 and MT2, while others are receptor independent involving direct intracellular actions. Initially, most of the experiments demonstrating the oncostatic role of melatonin were conducted in hormone-dependent tumors, mainly in breast cancer. However, there is growing evidence confirming that most kinds of cancer are susceptible of inhibition by the pineal hormone. The main goal of this work is to summarize the state of the art in a review of recent studies in which melatonin has been tested, either alone, or in combination with chemotherapeutic drugs on several types such as ovary, prostate, gastric, colorectal, pancreatic, hepatic and lung cancer, leukemia and glioblastoma. Many studies have shown that melatonin's co-administration improves the sensitivity of cancers to inhibition by conventional drugs, renders cancers previously totally resistant to treatment sensitive to these same therapies and reduces the toxic consequences of anti-cancer drugs while increasing their efficacy, contributing to the wellbeing of patients.

Objectives

There is growing evidence that melatonin, the hormone synthesized and released from the pineal gland, is associated with a lower risk of cancer since it mitigates tumor initiation, progression and spreading. Most of the anti-cancer effects of melatonin were initially characterized on estrogen dependent breast cancer models. However, many recent reports demonstrate a wide spectrum of action of the pineal hormone on other kinds of tumors. The main objective of this work is to review the state of the art of the current knowledge about melatonin oncostatic effects on ovary, prostate, gastric, colorectal, pancreatic, hepatic, lung cancer, leukemia and glioblastoma cancers in an attempt to identify which intracellular signaling pathways are more commonly affected by this indoleamine, particularly when is co-administered with conventional chemotherapeutic drugs.

Melatonin: synthesis and physiological actions

When Aaron B. Lerner and collaborators isolated and characterized the methoxy derivative of serotonin from bovine pineal extracts near the middle of the last century (1958), they would never have imagined the multiple functions that this molecule would eventually display. N-acetyl-5-methoxytryptamine, commonly known as melatonin, is an indolic hormone produced principally by the pineal gland in the human brain, but it is also produced in retina, thymus, bone marrow, respiratory epithelium, skin, lens, intestine and other sites[1]. Biochemists identified the steps in the synthetic pathway of melatonin, and the physiologists described one of the best-known function of the pineal now, that is the regulation of seasonal reproduction in photoperiod-sensitive mammals.

Research within the last 60 years, however, has revealed that melatonin presents a broad spectrum of physiological actions. Melatonin (Mel) has been characterized as an effective synchronizing agent in several physiological and pathological conditions. Among its effects, melatonin exerts modulation of immune defense responses, body weight and reproduction, tumor growth inhibition and anti-jetlag effects. Moreover, there is also evidence that melatonin could act as a potent antioxidant molecule, as a chemotoxicity reducing agent and a putative anti-aging substance.

Melatonin is a derivative of tryptophan. The synthesis of the pineal hormone from this amino acid takes account mainly in parenchymatous cells of the pineal gland in successive steps in which different enzymes participate. The rate-limiting enzyme is the Aryl-alkyl-amine N-acetyltransferase (AANAT), controlled by the light-dark cycle. The mammalian retinas have up to five highly specialized subtypes of melanopsin-containing retinal ganglion cells, or intrinsically photosensitive retinal ganglion cells (ipRGCs). These retinal ganglion cells directly detect light utilizing the photopigment melanopsin[2].

The oscillator in the melatonin rhythm generating system is located in the central biological clock, the suprachiasmatic nuclei, where the message related to the light environment is transferred. The suprachiasmatic nuclei is the master circadian oscillator and has an autonomous nature, as revealed in constant darkness, situation in which it

continues to drive rhythms slightly shorter or longer than 24 hours (depending on the species) in the pineal gland. The neural message arrives at the pinealocytes from the central nervous system (CNS) via the central and peripheral sympathetic nervous system. This enables the synchronization of the phases of the circadian clock with the light–dark cycle. Information relating to time passes from the CNS to the superior cervical ganglion and finally to the pineal gland. This pathway is stimulated during the night and, by contrary, light inhibits the activity of the superior cervical region. The release of norepinephrine from these sympathetic terminals induces the NAT activity and triggers the synthesis of melatonin. Melatonin synthesis is very sensitive to light exposure at night. Circulating levels of melatonin are low during the day and are dramatically abolished in animals exposed to constant light, whereas in animals living in continuous darkness, melatonin is synthesized following the endogenous rhythm driven by the suprachiasmatic nuclei.

Most of the actions of melatonin are due to its ability to bind to melatonin specific membrane receptors in target tissues. Two membrane receptors were pharmacologically characterized in neurons of the CNS and many peripheral tissues, subsequently cloned, and are currently identified as the MT-1 (Mel1a) and MT-2 (Mel1b) receptors. In contrast to the original assumption that membrane melatonin receptors were located on only a few cells, subsequent investigations have in fact localized them on many tissues, and they might well exist on the membranes of all cells. Both MT-1 and MT-2 are expressed in CNS, hippocampus, substantia nigra and ventral tegmental area. Besides, MT-1 receptors are found in retina, ovary, testis, mammary gland, coronary arteries, gall bladder, aorta, liver, kidney, skin, and the cardiovascular system (CVS); and MT-2 receptors can be found in brain, retina, and human pituitary gland. Additionally, a third putative receptor termed as MT-3 receptor is located in the cytosol of a few cells. As consequence of its location, MT-3 is not coupled to a G protein and exhibits low affinity for iodo-melatonin; it may be equivalent to quinone reductase 2.

MT-1 and MT-2 are both members of the superfamily of transmembrane, inhibitory G-protein coupled receptors, and they both share 60% homology. The binding of melatonin to its receptors impairs the activity of adenylate cyclase, therefore decreasing the intracellular concentration of cAMP. Some other second messengers such as cGMP, diacylglycerol, IP3 or PKC are also responsive to melatonin, depending on the cell type studied.

Considering the lipophilic nature of this indoleamine, some reports have pointed to the possibility that melatonin is able to bind nuclear receptors from the retinoid orphan receptor (ROR)-α and retinoid Z receptor (RZR) family. The superfamily members that reportedly bind melatonin include the RORα, RZRα, RORα2, and RZRβ. These nuclear receptors contain several domains in their structure: an NH2-terminal domain, a DNA binding domain, a ligand binding domain (in the COOH terminal), a zinc double finger, and a hinge region. The nuclear receptors may be differentially distributed among tissues and perhaps the model where they are best functionally described is the immune system.

Alternatively, other mechanisms of actions proposed for melatonin are not mediated by receptors, so, it seems that melatonin might have intracellular actions, binding to

cytosolic calmodulin, thus affecting calmodulin-dependent systems, such as cytoskeletal structural proteins, some intracellular soluble enzymes, and the estrogen receptor alpha (the cytoplasmic estradiol receptor that is overexpressed in most estrogen-responsive mammary tumors).

Additionally to its multiple actions mediated or not by its receptors as described above, melatonin also directly detoxifies reactive oxygen species (ROS) and reactive nitrogen species (RNS) by non-receptor mediated means. Like some other classic radical scavengers, major chemical mechanisms by which melatonin ensnares radicals include single electron transfer and hydrogen transfer. Consistent with its presumed high intra-mitochondrial concentrations, melatonin improves the activities of several respiratory chain complexes, thereby reducing electron leakage and free-radical generation. Moreover, metabolites of melatonin, such as cyclic-3-hydroxymelatonin (c3OHM), N1-acetyl-N2formyl-5-methoxykynuramine (AFMK), and N1-acetyl5-methoxykynuramine (AMK), provide additional protection against oxidative damage by the same mechanisms described above for melatonin and perhaps also by radical adduct formation. Although there are slight differences in their relative efficiencies, each of the four molecules are effective scavengers the highly reactive -OH at diffusion-controlled rates, regardless of the polarity of the environment.

To illustrate the importance of melatonin as an antioxidant throughout evolution, all the organisms investigated to date have the ability to synthesize this indoleamine. Additionally, besides being endogenously produced, melatonin is ingested in the diet of all animal species since this molecule is produced in plants. These dual routes ensure that melatonin is always available, unlike some other radical scavengers. Finally, at least in some animal and plant species, melatonin synthesis is upregulated under those circumstances in which free-radical generation has been dramatically increased. This induction in melatonin synthesis results in potentiation of its protective activities. Related to melatonin's capability of reducing molecular damage due to free radicals are its effects on anti- and pro-oxidative enzymes intracellular levels. These actions are prominent and highly reproducible but, mechanistically, not well investigated, although preliminary evidence suggests the involvement of the membrane receptors MT-1 and MT-2. The major anti-oxidative enzymes that are stimulated by melatonin under basal conditions include the intracellular superoxide dismutases (CuZnSOD and MnSOD), the selenium-containing glutathione peroxidases (GPX1, GPX2 and GPX3), and catalase (CAT). Conversely, under toxic conditions of high oxidative stress, these proteins are protected from free-radical damage, and their enzymatic activity is preserved. Melatonin also maintains the activities of enzymes that enhance intracellular levels of reduced glutathione (GSH), an important intracellular antioxidant. The pro-oxidative enzymes inhibited by melatonin include nitric oxide synthase, myeloperoxidase, and eosinophil peroxidase.

The physiological and pathophysiological actions of melatonin are numerous. Melatonin is best known for its mediation of circannual variations in metabolism and reproductive competence in photosensitive species, its ability to influence circadian processes, and its sleep-promoting activity. Each of these functions relies on the circadian message provided by the pineal-derived blood and cerebrospinal fluid melatonin rhythms that are transferred to cells that "have a need to know."

It has been speculated that all cells have the capability of synthesize melatonin, particularly in their mitochondria. This locally produced melatonin is for protection from free radicals of cells in the neighborhood via autocoid and paracoid actions (the locally produced pineal hormone is not normally released into the blood). Receptor-independent actions of melatonin and its metabolites relate to their ability to directly quench free radicals and non-radical, but toxic, species. Excessive free-radical generation is notoriously destructive and kills cells secondary to massive oxidative damage, which induces cellular apoptosis or necrosis. The loss of cells due to programmed cell death is a consequence and/or contributes to many diseases as well as to age-related deterioration.

Melatonin's ability to prevent molecular damage meted out by free radicals and the cellular mutilation becomes manifested since the indole protects against that destruction. Ultraviolet and ionizing radiation can produce such damage, as well as, ingestion of toxins, heavy metals, alcohol, smoking, prescription drugs, ischemia/ reperfusion injury, which occurs during a heart attack or stroke, severe inflammation, neurodegenerative diseases, and many other pathophysiological situations.

Similarly, aging is associated with the progressive accumulation of oxidative debris, which contributes to functional inefficiency of cellular processes, thereby inducing additional free radicals to be produced. Thus, aging becomes a vicious cycle. As molecular processes fail, oxidative damage accumulates, which leads to additional physiological collapse, further exaggerating the production of free radicals.

Melatonin, because of its ability to neutralize radicals, defers age-related dysfunction of several organs. Increased age leads to a gradual diminished melatonin production such that, in the elderly, the nocturnal melatonin rise in the circulation is either greatly attenuated or it no longer exists. The consequences of this reduction may be highly significant in terms of health. Normal circadian rhythms deteriorate, and, considering their importance for optimal health, the dysregulation of these rhythms, (such as the sleep/wake cycle) negatively affects organisms. Additionally, the loss of melatonin during aging contributes to the accelerated accumulation of oxidative stress due to the reduced availability of this important antioxidant. This presumably contributes to the progression of diseases that have a free-radical component, e.g., neurodegenerative diseases, cardiovascular disease, skin deterioration, and metabolic syndrome.

Melatonin and breast cancer

More than 90% of newly diagnosed cases of breast cancer are in their initially stages hormone-dependent, playing estradiol a crucial role in their genesis and progression. It is known that breast development at puberty and during sexual maturity is stimulated by estradiol, the most physiologically active hormone in breast tissue. Since 1896, when Beatson observed regression of advanced breast cancer after bilateral ovariectomy in premenopausal women, there is considerable evidence pointing to estrogens as mammary carcinogens. In 1978, when the role of lifetime excess estrogen exposure was gaining widespread recognition as a risk factor for breast cancer, Cohen, et al, proposed that diminished function of the pineal gland may increase risk of breast cancer by prolonging exposure to circulating estrogens. This hypothesis is based on several observations: i) The incidence of breast cancer is highest in countries in which pineal calcification is highest. ii) Patients taking chlorpromazine, a drug that raises melatonin levels, have lower rates of breast cancer. iii) in vitro data suggests that melatonin may have direct effects on breast cancer cells. iv) melatonin receptors are present on human ovarian cells, which suggests melatonin may have a direct influence on ovarian production of estrogen.

Tamarkin and collaborators reported that the amplitude of the nighttime peak of nocturnal plasma melatonin is diminished in women with estrogen receptor-positive breast cancer in comparison with estrogen-negative disease patients or healthy, matched controls. It is not totally clear yet, though, whether the diminished levels of the peak of melatonin is a consequence of a long-term failure of the gland, or rather a change that occurs at the time of breast cancer development. Other reports suggest an inverse correlation between nuclear grade and concentration of melatonin in the tumor tissue and a positive correlation between high levels of melatonin in the tumor and the estrogen receptor status, suggesting that melatonin concentration might constitute a good prognostic marker. Some additional observations are that breast cancer has a low incidence among blind women and an inverse association between breast cancer incidence and degree of visual impairment.

In 1987, based on Cohen's work, Richard Stevens suggested the hypothesis that women who are exposed to light at night will have higher rates of breast cancer. Studies have confirmed that circadian disruption or pinealectomy can lead to spontaneous tumor development and increased growth and metastatic potential of existing tumors. These observations, along with epidemiological evidence of higher cancer rates in night shift workers, has led the World Health Organization's International Agency for Research on Cancer to deem night shift work, "probably carcinogenic" to humans (Group2A).

Studies on pinealectomized rodents and those exposed to light at night suggest that reduced melatonin has a causal role in the growth and development of tumors. Exposure to light at night leads to accelerated growth of DMBA-induced mammary adenocarcinomas in rats. These results support the hypothesis that, in healthy premenopausal women, the exposure to light at night can result in enhancement of mammary oncogenesis through disruption of the circadian oncostatic actions of melatonin.

Studies performed by Schernhammer and collaborators addressing the relation between rotating night shift work and breast cancer risk in a cohort of premenopausal nurses, published that women who reported more than 20 years of rotating night shift work presented an elevated risk of breast cancer compared with women who did not work on these rotating night shift conditions[3]. Of note, confounding the meta-analysis is the lack of standardization in the definition of what constituted "night-shift work" between studies. Despite the lack of a unifying definition, the main conclusion is that there is an increased risk of breast cancer correlating with the increasing duration in years of working "night shifts" consistently documented across studies. Two of the most well-controlled studies assessed in the meta-analysis were prospective cohorts within the Nurses' Health Study (NHS) and Nurses' Health Study II (NHS II). Both studies were well controlled for confounders such as body mass index, reproductive history, family history, smoking status, age, use of hormones, and benign breast disease. While there was a small but measurable increase in risk of breast cancer in the groups working rotating shifts for 1–14 years and 15–29 years, the relative risk (RR) for women who worked more than 30 years of rotating shifts was a statistically significant 36% increase.

In a meta-analysis published in 2016, comprising more than 30 000 participants, the conclusion was that female flight attendants have a higher risk of breast cancer compared with the general population with an increase in the risk of developing breast cancer of a 44% for flight attendants versus controls from the general population. Of course, there are confounders that are unique to this population, such as time zone changes and radiation exposure. Nonetheless, these studies lend further support to the associated increased risk of work involving circadian disruption.

As pointed above, there are many studies on animal models *in vivo* and other experimental models *in vitro* supporting the hypothesis that melatonin antitumor effects in breast cancer are based on its antiestrogenic actions. The majority of animal studies have used as an animal model, the chemically induced DMBA mammary cancer in rats. In general, animals with enhanced pineal function or receiving melatonin as treatment, have an increase in tumor latency (the time transcurring between the administration of the carcinogen and the appearance of visible tumors, a lower tumor incidence, generally accompanied by a smaller size and lower number of tumors and a higher frequency of tumor regression in previously induced tumors. The animals treated with melatonin also showed a reduced expression of estrogen receptor alpha at the tumor level. Therefore, the results from these animal models suggest that melatonin may counteract the action of estrogens in tumor cells.

MCF-7 cells have widely used as an in vitro model of mammary tumors. The antiproliferative effects of melatonin on the breast cancer cell line MCF-7 have been studied for nearly thirty years. There is abundant evidence suggesting that the inhibitory action of melatonin on mammary cancer estrogen-positive cell lines is based on its ability to regulate either the synthesis of estrogens or estrogen signaling pathways. Melatonin indeed downregulates both the expression and activity of the enzymes necessary for the synthesis of estrogens from androgenic precursors, therefore acting as a Selective Estrogen Enzyme Modulator (SEEM). Melatonin inhibits aromatase, the main enzyme in estradiol synthesis. Melatonin also downregulates the expression and enhances the activity of steroid sulfatase (STS), 17β-hydroxysteroid dehydrogenase type

1 (17β-HSD1), enzymes that participate in forming active estrogens, and activates estrogen sulfotransferase (EST) that sulfonates estrogens to form biologically inactive estrogen sulfates.

Melatonin can also counteract the different actions of estrogens, thus functioning as a naturally occurring Selective Estrogen Receptor Modulator (SERM). The mode of action of the pineal hormone is different from those of other antiestrogenic molecules. Unlike tamoxifen, melatonin does not directly bind to estrogen receptor (ER). It has been described that melatonin decreases the expression of ERα in MCF-7 cells and interferes with estradiol-triggered transcriptional activation of many estradiol responsive genes through destabilization of the estradiol-ER complex, preventing its binding to DNA in both estrogen response element (ERE)- and activator protein 1-containing promoters. These actions of melatonin seem to be mediated by calmodulin, since calmodulin binds to ERα and melatonin behaves as a calmodulin antagonist. The pineal hormone promotes structural changes in the calmodulin-ERα protein complex, thus impairing its binding to estrogen responsive promoters. The effects of melatonin may also be explained in terms of binding to its specific membrane receptors MT-1 expressed in MCF-7 cells, therefore interfering with the estrogen receptor signaling. In mammary tumor cells, estradiol promotes adenylate cyclase activation, which results in higher cAMP cytoplasmic levels in a classical short-time second-messenger mechanism independent of gene transcription. High cAMP levels cooperate with long-time genomic effects of estradiol, thus enhancing ER-dependent transcriptional activation. The pineal hormone, through its specific binding to its membrane receptor MT-1, decreases the activity of adenylate cyclase, resulting in decreased cAMP levels. Therefore, melatonin and estradiol signaling pathways converge and have opposite effects over cAMP intracellular concentrations (*Figure 1: Cos et al., Cancer Detection and Prevention, 2006*).

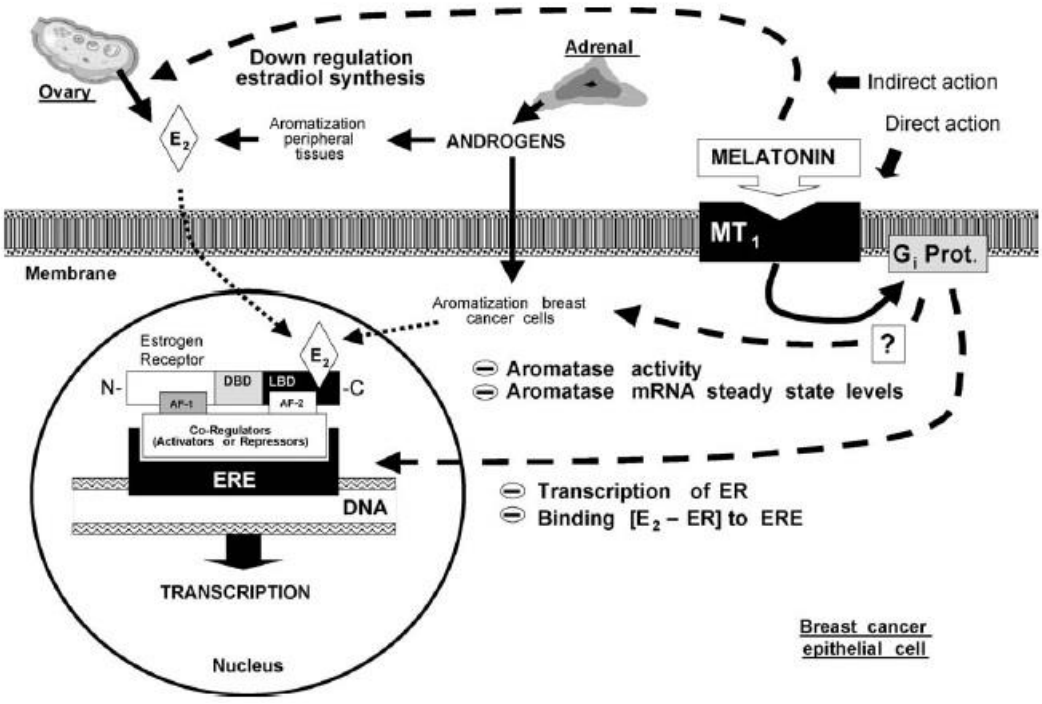

Figure 1: Estrogen-signaling pathway: a link between breast cancer and melatonin oncostatic actions (Cos et al., Cancer Det Prev, 2006)

Melatonin shows antimitotic actions on hormone dependent human cancer cell lines such as MCF-7. Under different culture conditions, melatonin is able to block the mitogenic effects of estradiol. Estrogens enhance cell proliferation and provoke cell cycle progression. The inhibitory action of melatonin in human breast cancer cell lines might be explained through the modification of the levels of estrogen-modulated proteins, several growth factors, and proto-oncogenes such as c-MYC, transforming growth factor (TGFα), Trefoil factor 1, also known as pS2, progesterone receptor (PGR), or the AP1 transcription factor subunit c-Fos. Additionally, the pineal hormone has been shown to decrease the motility and invasive capabilities of breast cancer cells (MCF-7) cells *in vitro*. This is partly due to melatonin's effects on cell surface adhesion molecules such as E-cadherin and β1-integrin[4]. These adhesion molecules allow for attachment of the cells within the extracellular matrix as well as to each other. Estrogen down-regulates these adhesion molecules, increasing the invasive potential of the cell. In tumor progression, down-regulation or even the loss of molecules involved in cell-cell recognition frequently occurs, leading to an invasive phenotype. Moreover, these events correlate with poor cell differentiation, and poor prognosis in cancer progression. Melatonin has been shown to increase the expression of these adhesion molecules in MCF-7 cells.

The antiproliferative effect of melatonin on cell cycle progression might also been explained by its effects on the expression of certain key proteins regulating the G_1-S cell cycle transition. Several studies have demonstrated that melatonin increases the expression of p53 and p21 in MCF-7 cells. The upregulation of these cell cycle regulators may be a crucial mechanism by which melatonin stops the progression through the cell cycle at the G_1-S transition. The accumulation of cells in G_1 forces them to enter G_0, inducing the cancer cells to undergo a higher differentiation. It is well known that melatonin inhibits cell proliferation and enhances cell differentiation.

Other observations contribute to explain the oncostatic effects of melatonin in hormone-dependent tumors. Melatonin can regulate the production and secretion of cytokines from epithelial malignant cells. These cytokines modulate the differentiation of fibroblasts placed in the next proximity of malignant epithelial cells. In addition, these cytokines stimulate estradiol synthesis in these fibroblasts and in proximal endothelial cells by increasing the expression and activity of aromatase.

The main cytokine produced by malignant cells is the vascular endothelial growth factor (VEGF). This factor plays a crucial role in angiogenesis. VEGF is released by tumor cells and binds to its receptor (VEGFR) triggering intracellular signaling pathways that induce endothelial cells to proliferate and migrate[5]. Melatonin can regulate the paracrine mechanisms responsible of the interplay between tumor epithelial cells and surrounding fibroblasts and endothelial cells. The pineal hormone downregulates VEGF production in estrogen-responsive breast cancer cells. As consequence, the levels of VEGF released are lower, and therefore, the number of cells synthesizing estrogens in the proximity of the malignant cells is decreased. Lower levels of estrogens and a reduced capability of formation of new vessels as a result of the presence of melatonin will diminish the tumor ability to spread and grow.

Other actions of melatonin include intracellular effects independent of estrogen-signaling pathways. Melatonin is a potent antioxidant, reducing reactive oxygen species with a resultant decrease in DNA damage. It has also been shown to inhibit the expression and activity of telomerase, the enzyme responsible for conferring immortality to cells, both in vitro and in vivo. Another cytostatic effect of melatonin can be explained since melatonin leads to a decrease in cellular uptake of linoleic acid. Reducing availability of linoleic acid results in an anti-proliferative effect through the resultant decrease in its metabolite 13-hydroxyoctadecadienoic acid (13-HODE), which serves as an energy source and growth-signaling molecule for proliferative pathways such as epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK) pathway.

Melatonin has also been shown to modulate immune function. One study showed extrapineal production of melatonin by lymphocytes increased the activity of interleukin-2 (IL-2) and IL-2 receptor system. Clinically, many trials have shown a synergistic effect wimth therapeutic IL-2 administration in various cancers. More generally, the systemic anti-inflammatory effects, as demonstrated in lower circulating levels of IL-6 and erythrocyte sedimentation rate (ESR) in patients taking melatonin, may affect both tumorogenesis and proliferative and metastatic pathways that are otherwise stimulated by inflammatory cytokines.

Melatonin: clinical trials

Much of the evidence supporting the use of melatonin in treating various cancers comes from clinical trials done by Paolo Lissoni of Italy. He has demonstrated a wide array of beneficial effects of high dose melatonin (10–40 mg daily before bed) in patients with advanced cancers of varying origin. Concerning women with breast cancer, this group have performed two trials on the use of high-dose melatonin in women with breast cancer.

In a pilot study by Lissoni in 1995[6], melatonin showed benefit in women who had not responded to tamoxifen (TMX) therapy alone. Fourteen women with metastatic breast cancer who had not had a clinical response to TMX were given 20 mg of TMX at noon and 20 mg of melatonin in the evening. A partial response, defined as CT-confirmed reduction of lesions by greater than 50%, was noted in 4 of 14 patients with a median duration of 8 months. Two of these 4 responders had singular lung lesions, one had pleural metastasis, and the fourth had skin metastasis. Stable disease, defined as no objective regression or increase less than 25%, was noted in 8 of 14 patients, 2 of whom had progressive disease on TMX alone. Of note, circulating levels of insulin-like growth factor (IGF-1) decreased in all patients on melatonin, with a significantly greater decrease in those who had a clinical response. Further, 6 of the patients enrolled, and 2 of the 4 responders were ER–negative, yet had previously been given TMX due to ineligibility for poly-chemotherapy approaches. This study was done prior to the advent of aromatase inhibitors, which would certainly be applied today to postmenopausal patients who have progressive disease on TMX. Nevertheless, this small trial

demonstrates the possible use of a nontoxic agent to enhance the effectiveness of an otherwise ineffective therapy.

The second breast cancer trial by Lissoni's group evaluated high-dose melatonin in women receiving epirubicin chemotherapy weekly, but who had to delay treatment due to the limiting toxicity of thrombocytopenia. Fourteen women with thrombocytopenia were given 20 mg per evening of melatonin for 7 days, prior to begin weekly epirubicin treatments. After 4 cycles, the induction phase of melatonin normalized platelets in 9 of 12 evaluable patients. There was no further platelet decline in these patients throughout the chemotherapy treatment. Tumor regression was achieved in 5 of 12 of the patients and no toxicities were noted. This small trial suggests that melatonin may enhance platelet production and decrease thrombocytopenia in breast cancer patients receiving epirubicin. Larger trials confirming this benefit to platelet-depleting drugs, including epirubicin, are needed in order to make such a conclusion.

The use of melatonin on a variety of cancers has been addressed in several clinical trials. There is a large number of works pointing to the potential benefits of melatonin if added together with chemotherapeutic agents. In breast, lung and gastrointestinal cancer patients, melatonin protected against thrombocytopenia, and stomatitis, asthenia and neuropathy were less recurrent in the group of melatonin-treated patients. It has also been described a protective effect of melatonin to hematopoietic progenitors against the toxic actions of anticancer chemotherapeutic drugs; for example, melatonin attenuates the damage to precursor blood cells caused by both radiotherapy and chemotherapy protocols.

Additionally, the pineal hormone increases 1-year survival and induces tumor regression rates in cancer patients with metastatic solid tumors with poor clinical status. In metastatic non-small cell lung cancer patients receiving only chemotherapy or with chemotherapeutic agents plus melatonin, both the overall tumor regression rate and the 5-year survival rate of those patients concomitantly receiving melatonin were better compared with those receiving only chemotherapeutic agents[7]. Moreover, chemotherapy was better tolerated in patients who also received the pineal hormone, pointing to melatonin as an adjuvant chemical able to ameliorate the effectiveness of chemotherapy in terms of both quality of life and survival of patients.

A recent review comprising data from 21 clinical trials (all the patients enrolled in the studies were bearing solid tumors), concluded that melatonin may serve a beneficial role in cancer patients who are treated with chemotherapy. In the clinical trials compiled in this study, the effect of melatonin concomitantly added in conjunction with chemotherapy or radiotherapy was evaluated, and supportive care, partial response, complete response, 1-year survival and chemotherapy-associated toxicities were assessed. Patients who received melatonin experienced substantial improvements, particularly in terms of tumor remission and 1-year survival rates and melatonin also ameliorated the side effects of chemotherapy.

There is growing evidence that melatonin interferes with cancer initiation, progression and spreading, and many cancers are susceptible to inhibition by melatonin. Work from many researchers point to melatonin as a molecule that has a great potential to be

useful as an anticancer chemical that has not the disadvantage of producing adverse effects[8]. As pointed earlier, several studies point to melatonin as an agent able to reduce the toxic consequences of anti-cancer drug, whereas their efficacy is increased. Many recent reports suggest that melatonin concomitantly administered with chemotherapeutic drugs improves the sensitivity of cancers to inhibition by these agents. Additionally, melatonin seems to play synergistic effects with a variety of chemotherapeutic drugs blocking cell proliferation and inducing cell death. The main goal of this work is to summarise the current knowledge regarding the interplay of melatonin and chemotherapy in many kinds of cancer apart from breast cancer.

Melatonin and ovarian cancer

Ovarian cancer (OC) is the second most common gynecologic malignancy in developed countries, and is considered the leading cause of death due to gynecological malignancy. OC is not just a single disease but many diseases, composed of a diverse group of tumors that can be classified accordingly to distinctive morphologic and molecular genetic features. Over 95% of primary ovarian malignancies are derived from epithelial cells; the remainder arise from other ovarian cell types (for example: germ cell tumors or sex cord-stromal tumors). The risk of developing ovarian cancer gets higher with age, thus, in women younger than 40, there is a low incidence. Most OCs develop after menopause. About 50% of patients with OC are 63 years of age or older, being the higher risk in women aged 70 and older. This illness is frequently diagnosed at younger ages among women with a hereditary ovarian cancer syndrome. The risk of OC reaches 2 to 3 percent in women with a BRCA1 gene mutation at age 35 and for those with a BRCA2 mutation at age 50. The typical age at diagnosis of OC in women with Lynch syndrome (hereditary nonpolyposis colon cancer) is 43 to 50 years old. OC produces few perceptible symptoms when localized to the ovary. Due to the asymptomatic nature of the disease in its early stages, most patients do not seek medical care until the disease has progressed beyond the ovaries into the abdomen and/or pelvis. Most OCs are diagnosed at an advanced stage.

Improvements in OC therapies have allowed a greater number of young women to survive; however, many of these patients suffer from ovarian failure or early menopause followed by the loss of reproductive function (tipically by a loss of primordial follicles). Cryopreservation is used to safeguard fertility in young patients undergoing chemotherapy or radiation, but unfortunately, this option is not accessible to all cancer patients. Novel therapies must be developed for integration into conventional treatment strategies to achieve improved clinical outcomes.

It is known for more than 50 years that melatonin daily injected in rats decreased the incidence of estrus and reduced the weight of ovaries. Melatonin and some of its metabolites exert a direct effect on the human reproductive system by influencing the function of the ovaries[9]. Melatonin receptors are present in human granulosa cells, rat antral follicles, and in the corpus luteum[10]. The description of a melatonin receptor expressed in human ovaries, and the demonstration of the binding of [^{3}H]-Mel in the ovaries of hamsters, rats, and humans suggests a direct influence of this hormone on the ovarian function and therefore, in estrogen synthesis[11]. More recently, MT1 expression in both ovarian tumors and several ovarian cancer derived cell lines (SK-OV-3, OVCAR-3 and IOSE 364) has been reported[12]. MT1 expression was higher in patients over fifty years old and correlated with Ki-67 antigen expression. Higher expression of MT1 was observed in the ER-negative SK-OV-3 cell line in comparison with the ER-positive OVCAR-3, suggesting that the antiproliferative effect of Mel on OC cells may be the result of a receptor-independent mechanism. Furthermore, endogenous melatonin levels decrease with age, but it is still unknown whether this is associated with a reduction in MT1 expression in the ovary.

Several recent studies have addressed the anti-tumor activity effect of melatonin on ovarian cell lines and animal models of OC. An investigation about the protective effects

of melatonin as an anti-proliferative agent and a cell cycle regulator has been conducted in the OC cell lines OVCAR-429 and PA-1[13]. In this in vitro study, these two cell lines were subjected to increasing dosages of melatonin for periods from 24 to 72 h. The results indicated that melatonin treatment reduced the survival and proliferation of OVCAR-429 and PA-1 cell lines in a dose- and time-dependent manner, but no significant results in the percentage of cells undergoing necrosis, apoptosis, or caspase 3 activation at melatonin concentrations ranging from 400 to 800 µM were observed. Importantly, exposure to melatonin resulted in an increase in the number of cells in the cell cycle G_1 phase parallel to a decrease in the number of cells in the S phase, likely via down-regulation of cyclin dependent kinases CDK 2 and CDK 4 gene expression and/or the up-regulation of p53 and p21. This is the first study demonstrating that CDKs down-regulation might explain, at least in part, the anti-cancer effects of the pineal hormone in ovarian cancer.

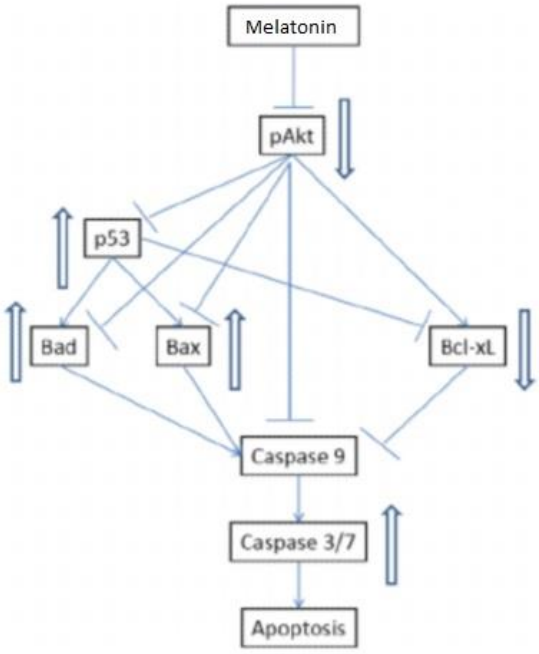

Figure 2: Mechanism/pathways for Melatonin-induced apoptosis in ovarian cancer cells (adapted from Haitao Luo et al., Food Chem, 2011)

Epidermal growth factor receptors 2 and 4 (Her-2 and Her-4) are closely associated with OC progression and metastasis, especially when the PI3K-AKT complex and MAPK are activated (Figure 2). Dysregulated Her-2 signaling is associated with an elevated risk of progression and death among women in both early and late stages of disease, and furthermore, high expression of Her-2 is associated with resistance to endocrine therapies and chemotherapies. In an in vivo model of OC on ethanol-preferring rats, the effect of melatonin on the expression of several mitogen activated protein kinases and epidermal growth factor receptors has been characterized. In this experimental model of animals bearing OC, melatonin therapy significantly downregulated Her-2, p38 MAPK, and p-AKT even in the presence of EtOH consumption. Overall, these findings indicate that the inhibition of Her-2 signaling by melatonin might be related to its anti-tumor effect in aggressive OC. This is the first study to demonstrate that melatonin therapy

negatively regulated the expression of Her-2, p38 MAPK, p-AKT, and mTOR, but not Her-4 and PI3K levels in OC of ethanol preferring-rats. These results point to melatonin to be considered as an adjuvant drug for OC chemotherapy[14].

Toll-like receptors (TLRs) are expressed on the surface of OC cells, but the signaling pathways triggered remain largely unclear. TLRs are cell surface sensors that can initiate pathways to stimulate cell proliferation and resistance to chemotherapy, as well as recruiting immune cells to provide support for cancer progression. It has been documented that TLR4 plays important roles in promoting the immune escape of several human cancer cells, including OC cells. It performs a protumor function contributing to hamper the efficacy of cancer therapies (e.g., paclitaxel). Furthermore, TLR4-mediated signaling is associated with the metastatic potential of tumor cells[15].

Melatonin negatively regulates the TLR4-mediated, but not TLR2-mediated signaling pathway in OC of ethanol preferring-rats. In fact, melatonin upregulated TLR2 and the combination of melatonin with EtOH caused downregulation of TLR2. Level of Myeloid differentiation primary response gene 88 (MyD88), a universal adapter protein used by TLRs to activate the transcription factor NF-kB was significantly reduced following melatonin therapy in OC. Consequently, melatonin significantly reduced the expression of NF-kB p50/p65. Furthermore, the NF-kB subunit p65 was reduced in both cytosolic and nuclear extracts, demonstrating that the pineal hormone may act through mechanisms implicated in NF-kB gene expression regulation and involving nuclear translocation of NF-kB p65. TRIF, another adapter of TLRs, responsible of MyD88-independent activation, was markedly downregulated by melatonin. The pineal hormone reduced the expression of immunosuppressive cytokines, such as TNF-α and IL-6. These cytokines usually act activating other molecules involved in the inflammatory response by recruiting macrophages and dendritic cells to the tumor. The presence of these cells contributes to tumor growth, angiogenesis, and metastasis[16].

Escape from apoptosis plays a crucial role in cancer progression and it has been largely reported in many articles that melatonin modulates apoptosis in multiple cancer subtypes. The role of melatonin on the pro-apoptotic and anti-apoptotic proteins in the same model of ethanol-preferring animals has been addressed. Melatonin positively regulated the expression of p53, BAX, and cleaved caspase-3 (all having pro-apoptotic effects), and down-regulated survivin (anti-apoptotic) in papillary OC cells. In contrast with other models, melatonin did not result in a down-regulation of the anti-apoptotic factor Bcl-2, however, melatonin enhanced fragmentation of DNA; together, the results suggest that melatonin induce apoptosis in OC cells of ethanol-preferring rats[17].

There is one study conducted in women in which serum from both healthy and OC patients were collected to measure the levels of melatonin. The results of the analysis demonstrated that the levels of melatonin were significantly lower in OC patients compared with healthy women. These results suggest that the lower serum levels of melatonin in OC might contribute to the pathogenesis of ovarian cancer[18].

The effect of melatonin and chemotherapeutic drugs in ovarian cancer was assessed for the first time in 2000. Primary cultures from seven ovarian were incubated with melatonin or with a pineal extract, in comparison with cispatinum or mafosfamide. The

assay was not very promising, since melatonin inhibited the growth of only two of the seven tumors assayed, whereas the pineal extract proved to be more effective than the pineal hormone, suggesting that other pineal products might be important. Surprisingly, in this work the combination of melatonin added in combination with the chemotherapeutic drugs was not tested[19].

Melatonin has been assessed in combination with cisplatin in OC cells lines sensitive (HTOA) and resistant (OVCAR-3) to the chemotherapeutic agent. Telomerase and proliferation were analyzed. Melatonin alone produced no antiproliferative effect on both chemo-sensitive and chemo-resistant cell lines. However, melatonin enhanced the anti-proliferative effect of cisplatin in the sensitive HTOA cells. OVCAR-3 cells did not respond to cisplatin alone, but when melatonin was included in the treatment, an arrest of proliferation was observed. Interestingly, in the OVCAR-3 resistant cells, telomerase activity was significantly lower in the cisplatin + melatonin group. These results suggest that melatonin might improve OC chemotherapy by enhancing cisplatin sensitivity in both sensitive and resistant to cisplatinum cell lines[20].

In SK-OV-3 cells, melatonin alone did not show a cytotoxic effect whereas cisplatin suppressed cell viability in a dose-dependent manner. Interestingly, combination of melatonin and cisplatin synergistically inhibited the viability of this cell line. Importantly, melatonin had a protective effect against cisplatin-triggered cytotoxicity in OSEN normal ovarian epithelial cells. The study concludes that co-treatment with both cisplatin and the pineal hormone increased the number of OVCAR-3 cells in sub-G_1, suggesting that melatonin increases cisplatin-induced apoptosis. This effect is likely mediated via the inactivation of ERK/p90RSK/HSP27 cascade since melatonin increased the cleavage of caspase-3, and melatonin and cisplatin synergistically inhibited the phosphorylation of ERK, and in parallel, a dephosphorylation of p90RSK and heat shock protein 27 were observed. Furthermore, the pineal hormone blocked both the co-localization and expression of p90RSK and HSP27. These results point to a potent synergistic action of melatonin and cisplatin on ovarian cancer cells, explaining at least in part the molecular mechanisms that might be involved in this effect[21].

Recently, the effect of melatonin and cisplatin in primordial follicle loss has been addressed. It is well known that premature ovarian failure is one of the major side effects of chemotherapeutic agents in young cancer patients. In an *in vivo* model, cisplatin and melatonin were administered to female mice. Cisplatin by itself significantly diminished the number of primordial follicles in the ovary, whereas melatonin protected against this loss. The pathway involved, at least in part, is the PTEN/AKT/FOXO3a signaling pathway. Melatonin prevented both the cisplatin-triggered inhibitory phosphorylation of PTEN and the cisplatin-induced phosphorylation of AKT, GSK3α, and FOXO3a, all of which participate inducing follicle activation. These results point to melatonin as a potential molecule able to protect ovaries and to preserve fertility during chemotherapy in young female cancer patients[22] .

There is just one report in which a combination of melatonin and tamoxifen was tested in a phase II clinical trial in untreatable metastatic solid tumor patients. The number of patients enrolled in the study is low, and the patients of ovarian cancer were only two. Both patients had lung metastasis and were treated with a chemo protocol that included

cisplatin, doxorubicin, and docetaxel. One of the patients showed progression of the disease and the other has stable disease. Although the global conclusion of the trial was that melatonin might have some beneficial effect in patients with solid metastasis, the study cannot conclude that melatonin has a positive effect when administered with chemotherapy[23].

Melatonin and prostate cancer

Implement of diagnostic tools such as PSA levels has helped early detection of new cases of prostate cancer (PCa), which has become one of the leading causes of cancer among males in most Western countries. And yet PC is an important health issue in most countries as there are no satisfactory therapeutic treatments for advanced hormone-refractory PCa. As prostate physiology is primarily controlled by androgens, initial strategies for prostate tumors include androgen ablation which triggers cell death in PCa cells. Unfortunately, throughout time (3-5 years) tumors become androgen independent through a not completely understood process. Highly penetrant inherited genes conferring the PCa phenotype have not been identified so far and environmental factors can account for the progression of the disease. Also, the disease clearly correlates with age and the prevalence of PCa is so high that it could be considered a normal age-related phenomenon.

Prostatic neuroendocrine differentiation (NED) is a major problem for oncologists and pathologists as its role in prostate cancer progression is uncertain. While "de novo" neuroendocrine (NE) carcinomas are quite rare and very aggressive (<1%), many clinical studies have suggested instead that an increase in NE cells within the epithelial tissue – usually referred to as NE-like cells – or NED occur. NE-like cells are non-proliferative, post-mitotic cells and usually display an androgen receptor (AR)-negative profile. NED has been traditionally associated with poor prognosis but NE-like population seem to be heterogeneous, and hence, their contribution to tumor growth is still a matter of debate.

Several groups have reported the antiproliferative effect of melatonin in PCa cell, and one of the mechanisms proposed includes NE-like trans differentiation. Therefore, prostate gland is a major target for the indole and its potential therapeutic role in PCa has been suggested. In 2016, it has been described that melatonin promotes NED and has a preventive role on the PCa progression, using a combination of LNCaP cell culture studies together with a well-known, PCa murine model, "transgenic adenocarcinoma of the mouse prostate" (TRAMP), which mimics the histopathological features during tumor progression of the human prostate[24].

Cell culture studies have shown that NE-like trans-differentiation can be triggered by multiple stimuli ultimately involving several signaling pathways. They compared the use of melatonin with other classical NE-like triggering stimulus, that is, the androgen deprivation, achieved using charcoal-stripped sera in the media.

Persistent ERK 1/2 activation was found in both, melatonin and androgen-deprived cells, which conforms the essential role of ERK activation during NED in PCa cells, resulting in a less proliferative and tumorigenic phenotype. Melatonin or androgen withdrawal didn't activate neither AKT nor STAT. Further insights showed point redox regulation as an essential upstream factor before ERK activation in melatonin-induced NED.

Combining microarray assay, Western blotting, and immunocytochemistry, they confirmed that melatonin blocked nuclear translocation of AR, thus confirming anti-androgenic actions of the indole. However, using a comparative genome microarray to check the differentially expressed genes in control, melatonin, or androgen-deprived

cells, some differences were found, suggesting a more complex role of the indole. They found potential regulatory effects on IGFBP3, which would deserve further attention because of being highly expressed in male tissues. The association between p-ERK activation and upregulation of IGFBP-3 observed has been reported in other cancer cells, which would explain later persistent p-ERK activation by melatonin and by androgen deprivation throughout the IGF1 pathway. The metabolic outcome of this regulation would explain the inhibitory effects on cancer cell growth.

Finally, melatonin prolonged the survival of TRAMP mice by 33% when given at the beginning or at advances stages of the tumor. Serum IGFBP3 was significantly elevated by the indole in early stages of the tumor, confirming *in vivo* the role of the IGF signaling in the oncostatic action of the indole.

Melatonin, gastric and colorectal cancer

Gastric cancer (GC) has become the fourth most common malignancy and the second in mortality of total cancer worldwide. It is estimated that in 2012 there were 951,600 new cases and 723,100 deaths from gastric cancer in the world. Despite the discovery of new molecular targets for therapeutic intervention for gastric cancer in clinical trials, drug resistance is still the major reason why failure occurs in gastric cancer treatment.

Colorectal cancer (CRC) is a common and lethal disease. Both environmental and genetic factors influence the risk of developing CRC. The incidence and mortality rates vary around the world. CRC is the third most commonly diagnosed cancer in males and the second in females, with 1.4 million new cases and almost 694,000 deaths estimated to have occurred in 2012. Rates are substantially higher in males than in females.

Globally, the incidence of gastric and CRC varies over 10-fold. The highest incidence rates are in Australia and New Zealand, Europe, and North America, and the lowest rates are found in Africa and South-Central Asia. These geographic differences appear to be attributable to differences in dietary and environmental exposures. Low socioeconomic status (SES) is also associated with an increased risk for the development of colorectal cancer. Potentially modifiable behaviors such as physical inactivity, unhealthy diet, smoking, and obesity are thought to account for a substantial proportion of the socioeconomic disparity in risk of new onset colorectal cancer. Fortunately, incidence rates in most other western countries have been stable or increased slightly during last years. In contrast, CRC incidence rates have rapidly increased in several areas historically at low risk, including Spain, and several countries within Eastern Asia and Eastern Europe. Death rates from gastric and CRC have declined progressively in the last decades. This improvement in outcome is due, at least in part, to detection and removal of colonic polyps, detection of CRCs at an earlier stage and more effective primary and adjuvant treatments. In contrast to these data, mortality rates continue to increase in many countries with more limited resources and health infrastructure, particularly in Central and South America and Eastern Europe.

As recently reviewed, melatonin has been shown to exert important protective effects in the gastrointestinal (GI) tract. There is at least 400 times more melatonin produced in the gastrointestinal tract than in the pineal gland; indeed, animal studies have established the presence of melatonin in the GI tract. Melatonin signaling may be autocrine, paracrine, and/or endocrine and the multiple roles ascribed to it are dependent on organ localization and physiological context[25].

Melatonin is synthesized from serotonin through two enzymatic steps, AANAT and ASMT. Analysis of gene expression data show that AANAT and ASMT are expressed in the small intestine epithelial. Both melatonin receptors, MT-1 and MT-2, are expressed in small intestinal mucosa. Many enterochromaffin cells throughout the GI tract also displayed MT-2. In the intestine, high concentrations of luminal melatonin, through its MT-2 receptor, increases bicarbonate secretion in the duodenum in response to acidic luminal contents, theoretically protecting the intestinal mucosa. Epithelial melatonin staining is strongest in the colon and rectum. These same sections also displayed very strong epithelial expression of both MT-1 and MT-2 receptors. Recently, it was shown

that very high doses of melatonin reduce epithelial paracellular permeability in rats and may prevent deleterious substances such as endotoxins from leaking in and causing inflammation.

The relationship between GI melatonin and circulating levels in the plasma appears to be bidirectional. It has been demonstrated that circulating melatonin and ingested melatonin accumulate in the GI tract. In the gut, melatonin appears to act as a functional antagonist of serotonin and dampens intestinal motility. Both, MT-1 and MT-2, have been found in the submucosal and myenteric plexuses, which have both parasympathetic and sympathetic input. In blood vessels, melatonin activation of MT-2 causes vasodilation, while MT-1 mediates vasoconstriction. There is also evidence that melatonin regulates endothelial permeability permitting leukocyte extravasation during an immune challenge.

Melatonin in gastric cancer

Melatonin is considered a promising agent in stomach cancer. In the gastric cancer cell line SGC-7901, the pineal hormone suppressed cell viability and stimulated apoptosis in a dose-dependent manner. At molecular level, apoptosis induced by melatonin was related to phosphorylation of HSP27. HSP27 is a member of the small heat shock protein (HSP) family, and it is upregulated in many cancers including ovarian cancer, colorectal cancer, and gastric cancer. Phosphorylation of this heat shock protein might be involved in apoptotic resistance. However, melatonin also increased PI3K/AKT activation. Treatment of cells with PI3K and p38 inhibitors prevents HSP27 activation and potentiates apoptosis. Therefore, it may be reasonable to think that activation of PI3K/AKT signaling upon treatment with melatonin increased HSP27 phosphorylation, which was resistant to melatonin chemotherapy. Taken together, all these results demonstrate that HSP27 plays a crucial role in apoptotic resistance. These findings are of potential pathophysiological importance for understanding the integration of melatonin-related signaling and further substantiate the molecular basis for clinical trials applying melatonin for the treatment of gastric cancer[26].

The melatonin nuclear receptor was included in the superfamily of nuclear receptors RZR/ROR, and at least subtypes (α, β and γ) have been describe. The existence of this receptor suggests that some of the immunomodulatory and antitumor intracellular effects of melatonin depend on nuclear signaling. RZR/RORγ plays an important role to inhibit the action of gastric cancer cell proliferation during hypoxia. Treatment of SGC-7901 cells with melatonin resulted in decreased expression of RZR/RORγ in hypoxic conditions, as well as SENP1, HIF-1α and VEGF. Furthermore, RZR/RORγ siRNA obviously abolished the inhibitory action of melatonin on proliferation and accumulation of HIF-1α and VEGF in hypoxic SGC-7901 cells via inactivation of RZR/RORγ. These results point to melatonin as a potent anticancer supplement for gastric cancer therapy[27].

Deepening in the mechanisms by which melatonin act through its RZR/RORγ receptor, it has been revealed that melatonin inhibits the growth of gastric cancer SGC-7901 cells in a dose- and time-dependent manner. This inhibition of tumor growth and formation of novel blood vessels, and suppression of tumor angiogenesis in nude mice is a consequence of a decrease in VEGF expression. Furthermore, melatonin treatment

reduced the expression of the melatonin nuclear receptor RZR/RORγ, SUMO-specific protease 1, HIF-1α and VEGF at transcriptional and translational levels within gastric cancer cells during tumorigenesis. From this work, it becomes clear that the anti-angiogenesis and antitumor activity of Mel against gastric cancer are related to downregulation of HIF-1α[28].

Very recently, in April 2017, it has been demonstrated in the same cell line, SGC-7901, that melatonin induces the levels of the phosphorylated forms of p38, and JNK and decrease the levels of phosphorylation of NF-kB. These result point to conflicting growth signals in response to melatonin. In one hand, the pineal hormone triggers apoptosis of gastric cancer cells, but, in the other hand, it stimulates signaling pathways that may inhibit the efficacy of melatonin in gastric cancer[29].

Melatonin and chemotherapy in gastric cancer

The pineal hormone was administered to gastric cancer patients either alone or in association with chemotherapy. Toxicity and efficacy of conventional chemotherapeutic combinations in the presence or absence of melatonin was tested. The main conclusions were that patients receiving melatonin presented a higher overall tumor regression rate and higher 2-year survival rate[30].

Calpains (intracellular signaling cysteine proteases) have been recently implicated in cancer development and progression, particularly in processes such as apoptosis and angiogenesis. Therefore, they are considered as potential anti-cancer targets. Melatonin has been tried in addition to tunicamycin, a drug that causes accumulation of unfolded proteins in the endopasmic reticulum leading to cell death by autophagy or apoptosis. Melatonin cooperates with tunicamycin activating calpain, dissociating C/EBPβ from NF-kB. In the presence of melatonin, COX-2 is downregulated, and the epithelial to mesenchymal transition markers E-Cadherin, Snail and Slug are reduced. These signaling pathways inhibited for melatonin correlated with the inhibition of peritoneal metastasis *in vivo*[31].

Melatonin and chemotherapy in oesophageal squamous cell carcinoma

Oesophageal squamous cell carcinoma (ESCC) is the sixth most frequent cause of deaths as consequence of cancer in the world. Among the new molecules considered as novel therapeutic alternatives, melatonin has been tested in combination with fluorouracil. Melatonin alone inhibited proliferation, cell migration, invasion, and triggered apoptosis of ESCC cells. At molecular level, melatonin suppressed the levels of phosphorylated forms of MEK, ERK, GSK3β and AKT. Fluorouracil alone had an opposite effect, activating ERK and AKT. Importantly, the effect of fluorouracil was reverted by melatonin and the pineal hormone enhanced cytotoxicity of this chemotherapeutic compound[32].

Melatonin in colorectal cancer

Several studies performed in patients bearing large intestine adenocarcinoma compared with a control group of healthy volunteers of comparable ages demonstrated that, although both groups presented a daily rhythm of melatonin secretion, there was a

significant decrease in the amplitude of rhythm and secretion of melatonin at nocturnal hours in the group of patients with cancer. These decrease in melatonin circadian rhythm occurred in all patients with colorectal carcinoma[33].

There is just one study addressing the effect of melatonin on the poorly differentiated colon carcinoma cell line RKO. Viability and migration experiments demonstrated that both cell proliferation and cell migration were inhibited after treatment with melatonin. Interestingly, the experiments of this work were performed with pharmacological doses of the pineal hormone, ranging from 0.5 to 2.5 mM, suggesting that lower levels of melatonin might be ineffective. At molecular level, they found that the anti-proliferation and anti-migration effects of melatonin correlate with lower expression levels of myosin light chain kinase (MLCK), lower levels of phosphorylation of its target, myosin light chain (MLC) and also with a reduced level of phosphorylation of p38. In this report, the use of ML-7, a specific blocker of MLCK, reduced the proliferation and migration of tumor cells, suggesting that MLC phosphorylation is involved in cell division, cell motility and cell invasion. The fact that melatonin treatment in RKO cells resulted in a decreased expression of MLCK and subsequently, lower levels of phosphorylation of MLC can explain, at least in part, the anti-proliferative effect of this indoleamine. Moreover, the addition of melatonin also inhibited p38 phosphorylation in a dose- and time-dependent manner. Phospho-p38 is the activated form of the protein, closely related to cell proliferation, migration, and cell survival in various colorectal cancer. Although the crosstalk between p38 and MLCK in RKO cells is not fully elucidated, the results of this work suggest that melatonin down-regulates phosphorylation of p38. Lower levels of phospho-p38 somehow reduces the expression of MLCK which in turn impairs the phosphorylation of MLC, leading to inhibition of migration and proliferation, suggesting that melatonin could be useful to retard the growth of colon cancer[34].

In the human colorectal adenocarcinoma cell line HCT116, melatonin upregulated the pro-apoptotic protein Bax and induced autophagy and apoptosis. The number of cells in S-phase of the cell cycle was half of the control after just two days. E and A cyclins expression was reduced, and p16 and p21 were increased. The main problem of many chemotherapeutic drugs administered in colon cancer is cardiotoxicity and neurotoxicity, and melatonin has a protective effect against these undesirable events, which points to the pineal hormone as a potential chemotherapeutic agent to be included in colon cancer treatments[35].

Melatonin and chemotherapy in colorectal cancer

Methotrexate is a chemotherapeutic drug effective in several cancers, including breast, leukemia, lymphoma, bladder and colorectal. This agent competitively inhibits dihydrofolate reductase, an enzyme necessary for tetrahydrofolate synthesis. Folic acid is necessary to obtain thymidine, required for DNA synthesis. One of the major problems of methotrexate is enterocolitis. Pretreatment with melatonin had a dose-dependent protective effect on methotrexate induced mucositis. The villi/crypt ratio, diminished by this agent, was almost restored by melatonin pretreatment, suggesting that melatonin could be beneficial in ameliorating methotrexate induced enteritis in humans[36].

One of the drugs currently in use in colorectal cancer is irinotecan, a derivative of campothecin that acts as an antineoplastic enzyme inhibitor specific for topoisomerase I. Irinotecan prevents relegation of the DNA strand by binding to topoisomerase I-DNA complex, and causes double-strand DNA breakage and cell death. Melatonin has been tested in combination with this drug, in patients with metastatic colorectal cancer progressing after one previous chemotherapy line containing fluorouracil. The percentage of disease control achieved in those patients receiving melatonin plus irinotecan was significantly higher than that observed in the ones receiving the drug alone. Diarrhea, one of the adverse side effects, was reduced in the group receiving melatonin[37].

The effect of melatonin in combination with ursolic acid in SW480 and LoVo colon cancer cells has been addressed. Ursolic acid is a pentacyclic triterpenoid carboxylic acid, that inhibits the proliferation of various cancer cell types by inhibiting the STAT3 signaling pathway. Combination of both molecules significantly enhanced inhibition of cell proliferation and migration and increased induction of apoptosis. At molecular level, the combined treatment induced the release of cytochrome c into the cytosol, induced cleavage of caspase and PARP, and reduced the expression of MMP9 and COX-2. Finally, NF-kB binding and p300 recruitment to COX-2 promoter was abrogated. These results demonstrated that melatonin enhances the antiproliferative and proapoptotic actions of ursolic acid in colon cancer cell lines[38].

There is a very recent report, published in March 2017, in which the effect of melatonin combined with fluorouracil as an anti-cancer combination in colon tumors has been tested. Again, cell proliferation, colony formation, migration and invasion of colon cancer cells was impaired. Melatonin has a synergic effect with fluorouracil triggering caspase/PARP dependent apoptosis. Combination of both molecules reduced the phosphorylation of PI3K, AKT and NF-kB (Figure 3). This last factor, NF-kB, was translocated from the nuclei to the cytoplasm, impeding the binding to iNOS promoter, repressing the iNOS signaling pathway[39].

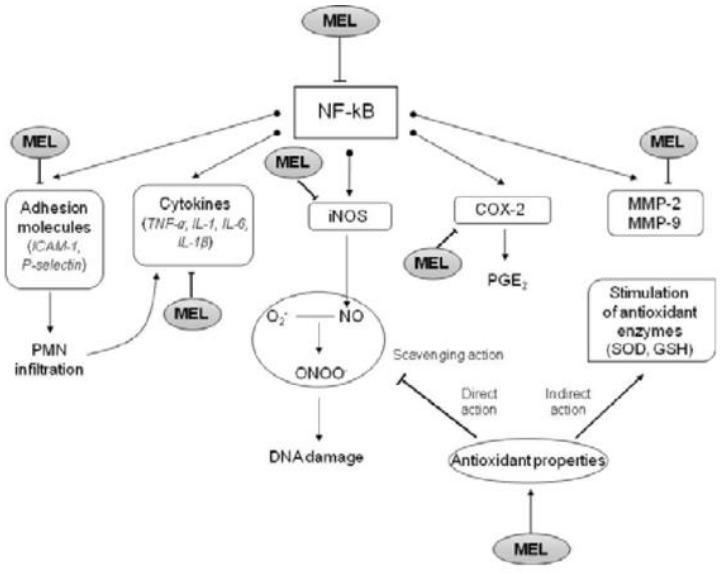

Figure 3: Proposed mechanisms implied in the anti-inflammatory and anti-oxidative effects of melatonin (Motilva et al., Journal of pineal research 2011*)*

Melatonin and pancreatic cancer

Pancreatic cancer (PC) is one of the leading causes of cancer mortality in developed countries and one of the most lethal malignant neoplasms across the world. The two main tumor types of pancreatic cancer are adenocarcinoma (that accounts for about 85% of cases), and pancreatic endocrine tumors (which correspond to less than 5% of all cases)[40].

Based on the GLOBOCAN 2012 estimates, PC causes more than 331.000 deaths per year, ranking as the seventh leading cause of cancer death in both sexes altogether. The estimated 5-year survival rate for PC is less than 5%. One of the principal reasons for this serious situation is the peritoneal dissemination that results in early metastasis. In many cases, when patients are diagnosed, the malignant tumor has become already unrespectable. The incidence and mortality of PC worldwide directly correlates with age and was slightly more frequent in men than in women. In the past decades, pancreatic cancer mortality has been increasing in both genders (for example, in the United States, European countries, Japan, China).

The causes of PC are still insufficiently known, although certain risk factors have been identified, such as cigarette smoking, positive family history and genetics, diabetes mellitus, obesity, dietary factors, alcohol abuse or physical inactivity. Understanding the epidemiology of PC could be the key to elucidate the etiology of pancreatic tumors and thus the cornerstone of developing a prevention strategy.

Several studies suggested that melatonin could be produced in pancreas as deduced from the fact that the enzymes necessary for production of melatonin are expressed in both the gastrointestinal tract and pancreas tissue. Melatonin immunoreactivity was later found in pancreatic islets. Gene expression of enzymes involved in the synthesis of melatonin, AANAT and ASMT have been detected in rat and human pancreatic acinar cells. It has also been shown that melatonin can influence transcription factors involved in insulin secretion in the pancreas in a receptor-dependent manner. MT-1 and MT-2 expression was also demonstrated in pancreatic Langerhans islets, which agrees with the newly described role of the pineal hormone in regulating circulating glucose levels via insulin and glucagon secretion.

The MT-2 receptor was found to be the dominant expression, which is a different result than that obtained in previous studies of mRNA expression in human islets where MT-1 expression was suggested to be higher than MT-2. Notably, decreased melatonin secretion is reported to increase the risk of developing type 2 diabetes, as well as genetic variants of MT-2 that lead to impaired melatonin signaling. MT-1 expression varied from strong to absent in all sections available, and the reason for this variation is not clear[41].

An explanation of melatonin anti-cancer properties based on compilation of findings from many studies indicate that melatonin could modulate the process of pancreatic oncogenesis by regulating different processes. The most well characterized are: a) Melatonin, through its role as a direct scavenger of radical oxygen and nitrogen species (ROS and RNS) and also through activation of antioxidant enzymes, can effectively protect the pancreatic tissue against oxidative stress and inflammatory damage. b) In

PANC-1 cells (derived from pancreatic carcinoma) pharmacological doses of melatonin alter the Bax/Bcl-2 protein balance, and stimulate the expression of caspase-9 and caspase-3, thus inducing the activation of the mitochondrial pathway of apoptosis. The pineal hormone, at low concentrations, trigger the production of anti-apoptotic heat shock proteins: HSP27, HSP70, and HSP90, therefore preventing the activation of caspase-3. c) Melatonin inhibits the production of vascular endothelial growth factor (VEGF) therefore reducing both angiogenesis and proliferation of endothelial cells. d) Melatonin strengthens the immune defense system of the organism via activation of peripheral effector T cells and suppression of T regulatory cells. e) In in *vivo* animal models, melatonin has been found to potentiate the cytotoxicity of chemotherapeutic drugs, thus reducing the adverse no desirable side effects of chemotherapy and therefore decreasing morbidity. These findings suggest that melatonin could be potentially taken into consideration as an adjuvant in the treatment of pancreatic cancer, although the effect of melatonin on apoptosis requires further investigation[42].

A previous study also provided evidence that melatonin interferes with the Bax/Bcl-2 protein balance and stimulates caspase-9 expression in human PANC-1 cells. The strongest signal of these pro-apoptotic proteins was obtained at very low doses of melatonin. Pretreatment with the specific melatonin receptor antagonist luzindole alone or prior to the addition of melatonin reversed the stimulatory effect of the pineal hormone on Bax, Bcl-2 and caspase-9 protein expression in PANC-1 cells, strongly suggesting that this effect is mediated through binding of melatonin to its membrane receptors[43].

In a recent report, it has been characterized the protective role that melatonin exerts in pancreatic tissue against the acute damage, and the molecular mechanisms involved in this beneficial effect could be related, at least in part, to the stimulation of Heat Shock Proteins (HSPs) in pancreatic PANC-1 cells. Melatonin induced the synthesis, phosphorylation, and nuclear translocation of HSP27 and caused overexpression of HSP70, HSP90αβ in the PANC-1 cultures, factors that are all involved in the modulation of cell proliferation, cell cycle regulation and protection of cells against damage. This was in opposition to the ability of this indolamine to stimulate the pro-apoptotic pathway on mitochondrial level and to activate procaspase-9 in pancreatic carcinoma cells[44].

Despite this, results have evidenced that melatonin failed to activate executioner of apoptosis: caspase-3, and prevented DNA fragmentation in PANC-1 cells. It is assessed that the changes in HSP27, HSP70 and HSP90 expression in human pancreatic carcinoma cells PANC-1 are subjected to N1 -acetyl-N2 -formyl-5-methoxykynuramine (AFMK) or Lkynurenine (L-KYN), the end products of the melatonin and L-tryptophan metabolic pathway. It has also been revealed that the application of MT1/MT2 receptor antagonist (luzindole) significantly reversed AFMK induced stimulation of anti-apoptotic HSPs production in PANC-1 cells. Similarly, the use of an inhibitor of 5-HT3 receptor resulted in the reduction of this melatonin metabolite activity.

Akin to other solid tumors, an adequate blood supply is the key factor in the tumor development of pancreatic cancer, through angiogenesis at both the primary and secondary cancer sites. In PC cells, both VEGF and VEGF receptors (VEGFR) are

overexpressed. VEGF promotes cancer growth, dissemination, and metastasis, and its expression level is positively correlated with the prognosis of pancreatic cancer in diagnosed patients or animal models. Therefore, VEGF-targeted therapeutic agents are a pivotal research focus and constitute a prospective strategy in the treatment of diseases, especially cancer. It has been demonstrated that melatonin, at pharmacological concentrations significantly inhibited VEGF production in PANC-1 cells, pointing to a possible anti-angiogenic effect, in agreement with previously described direct and indirect anti-angiogenic actions of melatonin on other tumors. After melatonin administration, a significant decrease of VEGF concentration in the culture was observed, in parallel with an evident inhibitory effect of melatonin on VEGF mRNA expression. This observation suggests that melatonin could be useful to treat pancreatic cancer, because of its anti-angiogenic actions[45].

A more recent study showed that melatonin inhibited cell viability, suppressed colony formation and reduced cell migration and invasion and induced cell apoptosis in MIA PaCa-2 cells. Results showed that melatonin treatment inhibited NF-κB p65 activation. Moreover, melatonin treatment activated the mitogen-activated protein kinase pathways (c-jun N-terminal kinase and extracellular-regulated kinase 1/2), which increased Bax protein expression and caspase-3 cleavage and decreased Bcl-2 protein expression[46].

Melatonin and chemotherapy in pancreatic cancer.

The first work trying to solve whether melatonin can be helpful when added to chemotherapy in pancreatic cancer was performed in Syrian hamsters. Melatonin was compared to celecoxib, a specific COX-2 inhibitor. Pancreatic cancer was chemically induced in the animals and melatonin and/or celecoxib were administered during the induction, post-induction, or both. The presence of tumor nodules and reduction of glutathione, superoxide dismutase and catalase, correlated with lower survival, was measured in all the experimental groups. The results obtained show that melatonin had a more potent beneficial effect than celecoxib since the number of tumor nodules in pancreas and splenic areas was lower, the oxidative stress was reduced and less animals died when melatonin was included in the treatment. Importantly, the administration of both compounds at the same time exerted a synergistic effect when given during the induction phase[47].

Using the same animal model of PC, melatonin was tested in combination with capecitabine. This antimetabolite is a pro-drug that is activated in the tumor cells by enzymes that convert capecitabine into fluorouracil, which inhibits DNA synthesis and slows the growth of tumor tissues. After chemical induction of poorly or moderate differentiated PC, melatonin and capecitabine were administered to the hamsters. Only 10% of animals simultaneously receiving both compounds showed a well differentiated adenocarcinoma, a percentage much lower in comparison with 66% of animals receiving capecitabine or 33% receiving melatonin. This positive effect was associated with lower lipoperoxide levels and higher levels of antioxidant activity in pancreatic tumor tissues[48].

Melatonin in association with chemotherapeutic drugs has been also tested cultured PC cell lines and has proven to be useful in some studies performed *in vitro*. The effect of

melatonin in addition with one of either cisplatin, doxorubicin or capecitabine was addressed in the pancreatic tumor cell line AR42J. All the combinations tested induced a stop in cell proliferation and higher levels of cytotoxicity in comparison with each of the three chemotherapeutic drugs alone. When melatonin was added, a higher percentage of cells entered into apoptosis, showed depolarization of the mitochondrial membrane and also presented higher levels of reactive oxygen species, proving that melatonin, in this cell line, had a powerful synergistic effect with chemotherapeutic agents[49].

An inhibitory growth action of melatonin on PC was demonstrated both in vitro and in vivo, in the cell line SW-1990, and in nude mice with implanted xenografts respectively, supporting the previous researches showing that melatonin had anti-cancer actions on pancreatic tumors. Oxidative stress plays an important role in carcinogenesis of PC, and the anticancer properties of melatonin are thought to be related to its direct or indirect antioxidative capacity in PC. Apoptosis and necrosis could be two possible mechanisms to explain the growth-inhibition of melatonin against PC cells, besides its antioxidant effect. Modulation of Bcl-2/Bax balance, via down-regulation of Bcl-2, may be a plausible reason for the pro-apoptotic and pro-necrotic effect of melatonin treatment in SW-1990 cell line. Gemcitabine is currently used as part of the standard chemotherapy protocols for pancreatic cancer, especially for the resectable ones. The median survival duration was only 5.7–6.3 months in those animals receiving just gemcitabine monotherapy. Combination therapies including gemcitabine with other cytotoxic agents, such as irinotecan and oxaliplatin, induced improved response rates over gemcitabine monotherapy, however randomized phase III trials of these combinations have shown no significant survival benefits in human patients. Interestingly, both *in vitro* and *in vivo* experiments showed that melatonin had similar anti-tumor effects as gemcitabine against PC cells. After the combined treatment with melatonin and gemcitabine, the tumor growth in nude mice was significantly inhibited in comparison with gemcitabine monotherapy, once again suggesting that melatonin might be a possible agent benefiting the chemotherapy of PC clinically[50].

In an attempt to further explore the actions of melatonin in combination with gemcitabine, several pancreatic tumor cell lines (AsPc-1, MiaPaCa-2 and Panc-28) and nude mice with pancreatic tumors bearing xenografts were treated with both compounds. In the cell lines tested, melatonin enhanced the effects of gemcitabine on cell proliferation and invasion, action that was independent of melatonin membrane receptors. Mice treated simultaneously with both agents has greater reductions in tumor size compared to animals treated only with gemcitabine. The most important finding of this work is that melatonin suppresses IKBα phosphorylation, which in turn results in an inhibition of NF-kB. Genes that respond to NF-kB show lover levels of expression and these molecular changes seem to reverse gemcitabine resistance in pancreatic tumors[51].

Melatonin and hepatocellular cancer

Primary liver cancer is a major health problem in many areas of the world; it ranks as the fifth most common cancer in males and the ninth most frequent cancer in females. Additionally, hepatocellular carcinoma ranks as the second most common cause in males and the sixth most common cause in females of cancer-related death. Hepatocellular carcinoma (HCC) represents almost 90% of primary liver cancer cases. Of the 782,000 new cases of HCC reported globally in 2014, at least 50% were documented in China, according to the most recent GLOBOCAN statistics[52].

Therapy decisions for HCC must take into consideration many factors including disease-related and patient-related factors. Patient-related factors of relevance include performance status and liver functional status while disease-related factors include number and size of hepatic lesions as well as presence or absence, and extent of vascular or extrahepatic involvement. Surgery, radiotherapy, and chemotherapy are the major treatment modalities, but all of them could induce certain undesirable side effects such as hepatotoxicity and necrosis.

According to Barcelona Clinic Liver Cancer (BCLC) staging and therapeutic algorithm, patients with very early/early disease should be treated with potentially curative options (e.g. resection, transplantation, or local ablation) while patients with intermediate stage disease should be managed with trans arterial (chemo)embolization. Patients with advanced disease (including those with portal invasion, or nodal or distant metastases) should be managed with systemic therapy. For those patients who have unresectable HCC, chemotherapy is indicated. To date, sorafenib is the compound showing better results as part of chemotherapy and it is the only drug approved for the systemic treatment of HCC.

The effect of melatonin administration on hepatic cancer was first investigated in HepG2 human hepatocarcinoma cells. Melatonin, at pharmacological concentrations ranging from 1 to 10 mM, resulted in a dose dependent reduction of cell number. This effect was correlated with a pro-apoptotic action of melatonin, since the pineal hormone triggered apoptosis, increasing caspase-3 activity and proteolysis of PARP. Apoptosis was accompanied by a reduction in the percent of cells in G_0/G_1 and increase in G_2/M indicating the cell cycle arrest in G_2/M. Reduced cell proliferation was accompanied by higher levels of p53 and p21, and also by up-regulation of JNK, p38, Bax, caspase 8, caspase 9 and cytochrome c release. All these molecular effects described, that eventually result in cell death and cell cycle arrest, proved the oncostatic effect of the pineal hormone on hepatocarcinoma therapy[53].

The protective effect of melatonin as an anti-oxidant agent on hepatic cancer was addressed using H4IIE hepatoma cells as an *in vitro* model. The idea was to test whether or not melatonin was able to trigger in these cells various mechanisms to reverse the deleterious effects of reactive oxygen species (ROS). In H4IIE cells, H_2O_2-induced activation of the extracellular signal-regulated protein kinases ERK1/2 and p38 MAPK, and some of their downstream protein effectors, were strongly weakened by melatonin, as well as H_2O_2-induced phosphorylation of Akt and the Akt substrate mTOR, and eIF4E-binding protein 1 (4E-BP1), mainly via preventing Ras activation

(Figure 4). The main conclusion is that melatonin is able to triggered different protective mechanisms against ROS induced damage in hepatoma cells, preventing many of the H(2)O(2)-induced alterations in the MAPK and mTOR signaling pathways through inhibition of Ras[54].

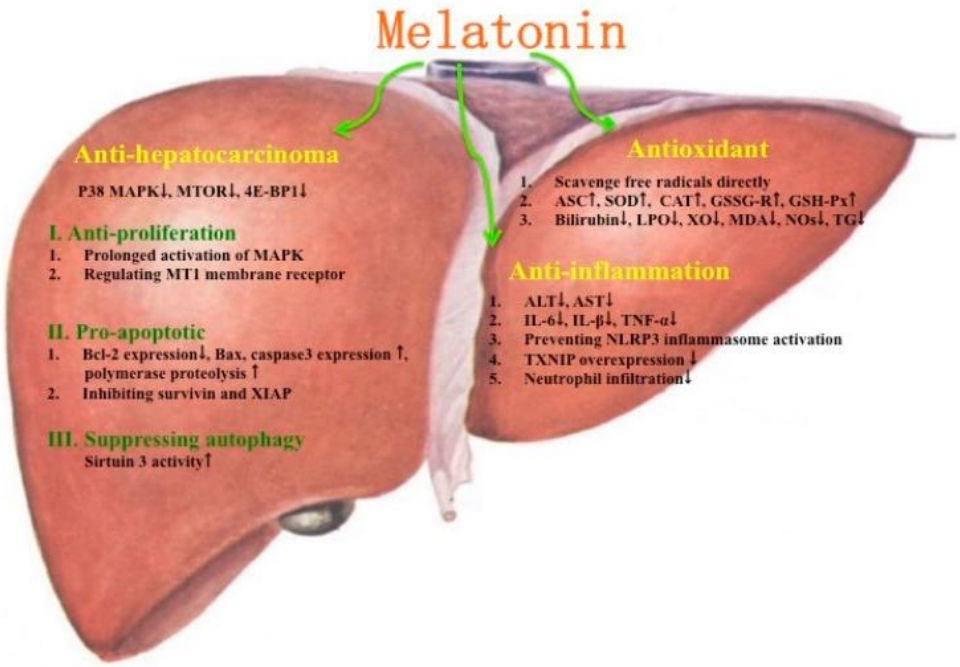

Figure 4: Effects of Melatonin on Liver Injuries and Diseases (Jiao-Jiao Zhang et al., International Journal of Molecular Sciences 2017)

As already mentioned in this review, some of the actions of melatonin on cancer cells can be explained since its specific melatonin receptors are expressed on them. In HepG2 human hepatocarcinoma cells, melatonin, at micromolar concentrations, significantly reduced cell viability. The levels of cAMP were significantly diminished after treatment with the pineal hormone, and this effect was partially blocked by luzindole, an MT-1 receptor antagonist. Melatonin increased the expression of several MAPKinases such as p38, ERK and JNK. Interestingly, ERK activation was completely abolished by luzindole. These results suggest that melatonin, in hepatocarcinoma HepG2 cells, regulates ERK by controlling the second messenger cAMP intracellular levels[55].

The effect of the pineal hormone was further studied in these same model (HepG2 cells) in order to determine the anti-angiogenic effects of melatonin. Hepatocarcinoma growth and spread depends on angiogenesis, and the main factor involved in this process is the vascular endothelial growth factor (VEGF). Melatonin at pharmacological concentrations (1 mM) decreased secreted levels of VEGF by HepG2 cells, and also prevented HUVEC (endothelial cells) tube formation under hypoxia. Prevention of tube formation seems to be associated with a down-regulation of the hypoxia inducible factor HIF-1α. These results confirmed an anti-angiogenic role of melatonin in hepatocarcinoma[56].

HCC is one of the most lethal cancers because of its metastatic potential. The capability of invading and spread outside the initial focus is related to extracellular matrix degradation by MMPs. Therefore, targeting MMPs by both synthetic and natural

inhibitors may be a novel strategy in HCC therapy. Melatonin exhibits anti-invasive and anti-metastatic properties in a variety of tumors by suppressing the enzymatic activity of MMP-9. In HepG2 cells, pharmacological concentrations of melatonin impaired MMP-9 gelatinase activity and effectively blocked cell invasion and motility through a simultaneous downregulation of MMP-9 gene expression and upregulation of the MMP-9-specific inhibitor metalloproteinases (TIMP)-1. In parallel, melatonin significantly suppressed IL-1β-induced NF-κB translocation and transcriptional activity. As conclusion, melatonin not only inhibits hepatocarcinoma cell proliferation, but also impairs angiogenesis, invasion and metastasis[57].

Apoptosis resistance in HCC is an important factor in hepatocellular carcinogenesis and tumor progression, and causes resistance to conventional treatments. Therefore, pro-apoptotic ability might be a key factor in treating HCC. Melatonin has shown its pro-apoptotic effect in hepatocarcinoma in many studies, some of them above commented. Inhibitor of apoptosis proteins (IAPs) expression has been related to apoptosis resistance. Four members of IAPs (cIAP-1, cIAP-2, survivin, and XIAP) were overexpressed in human HCC tissue. Inhibition of the growth of HepG2 and SMMC-7721 cells and promotion on apoptosis, accompanied by the down-regulation of survivin and XIAP were found after melatonin treatment. Moreover, cIAP-1, survivin and XIAP, were related to the co-expression of COX-2 in human HCC cells, and melatonin also decreased COX-2 expression and prevented Akt activation in HepG2 and SMMC-7721 cells. These results indicate that melatonin overcomes apoptosis resistance via the COX-2/PI3K/AKT pathway[58].

Very recently, some more research has been performed in order to further elucidate the ways melatonin works to achieve apoptosis. Activating transcription factor (ATF-6) is selectively blocked by melatonin in HepG2 cells. Blocking of ATF-6 leads to COX-2 down-regulation and results in a decrease of the ratio Bcl-2/Bax. The employ of an inhibitory ATF-6 siRNA enhanced apoptosis in HepG2 cells treated with tunicamycin (an endoplasmic reticulum stress initiating unfolded protein response pathway. Importantly, these findings described in the hepatocarcinoma cell line used in this study were confirmed in clinical hepatocellular carcinoma patients, indicating that melatonin acts a selective ATF-6 inhibitor, sensitizing human hepatoma cells to ER stress induced apoptosis[59].

Melatonin and chemotherapy in hepatocarcinoma

In chronological order, the first study investigating whether melatonin has synergistic effects with a chemotherapeutic drug in hepatocarcinoma was published in 2010, and it was performed in two human hepatoma cell lines: Bel-7402 and HepG2. The drug chosen to be combined with melatonin was doxorubicin. As expected, melatonin alone had a dose dependent inhibitory effect on cell proliferation. Importantly, when combined with doxorubicin, the pineal hormone significantly enhanced the inhibitory effect of this chemotherapeutic drug on growth inhibition and induction of apoptosis. The cooperative effect in induction of apoptosis was consequence of a diminished expression of Bcl-2 in parallel with an increase in the levels of both Bax and caspase-3[60].

The same group, in 2013, investigated the influence of melatonin on endoplasmic reticulum stress-induced resistance to chemotherapeutic agents, including doxorubicin. Tunicamycin, an endoplasmic reticulum stress inducer was administered to HepG2 and SMMC-7721 human hepatocellular cell lines. Pre-treatment with this agent dramatically reduced the rate of apoptosis in response to doxorubicin. Melatonin prevented the anti-apoptotic effect of tunicamycin. The levels of phosphorylated AKT were elevated in response to tunicamycin and strongly reduced in presence of melatonin. The pineal hormone also induced high levels of C/EBP-homologous protein and reduced the levels of survivin, a protein involved in inhibition of apoptosis. These findings suggest that inhibition of the PI3K/AKT signaling pathway by the pineal hormone is able to reverse ER stress-induced resistance to doxorubicin, through an increase in the levels of C/EBP-homologous protein and a downregulation of the levels of surviving in hepatocarcinoma cell lines[61].

Another important aspect to be considered when administering melatonin together with chemotherapy, apart from its synergic effects on cytotoxicity in cancer cells, is its ability to protect against toxicity in normal tissue. The protective effects of melatonin against hepatotoxicity induced by cyclophosphamide were evaluated in mice. The main effect of this agent is to form a metabolite: phosphoramide mustard, that forms inter-strand and intra-strand crosslinks within DNA, acting mainly on those cells actively dividing.

Histological studies after simultaneous treatment with melatonin and cyclophosphamide were conducted in mice. Those animals receiving cyclophosphamide presented severe hepatic damage, including abundant necrotic hepatocytes, severe inflammation in the portal space and general lymphocytic infiltration in hepatic tissues. By contrary, mice receiving a combination of cyclophosphamide and melatonin, presented less histopathological abnormalities in normal liver tissues, suggesting that the pineal hormone could be a good candidate to be used in hepatocarcinoma, not only for its enhancement of apoptosis induced by chemotherapeutic drugs, but also for its ability to protect patients from chemotherapy induced hepatotoxicity[62].

Cisplatin, a drug that interferes with DNA replication, is commonly employed in cancer treatment, but its use is restricted because of its toxicity and chemo resistance. The concomitant use of cisplatin with melatonin can be an option to improve its anti-cancer actions lowering at the same time its side effects in hepatocarcinoma treatment.

The experiments performed in four different hepatocarcinoma cell lines proved that melatonin enhanced the anti-proliferative effects of cisplatin in all of them. This anti-proliferative action is due to an increase in cisplatin mediated apoptosis. Some of the mechanisms involved have been characterized. Thus, combination of cisplatin and the pineal hormone enhanced the cleavage of PARP, and both caspase-3 and caspase-9. When melatonin was used, a diminished expression of Bcl-2 and p-IKKα/β was observed. The nuclear translocation of NF-κB p50/p65 proteins was abolished, and the binding of p65 to COX-2 promoter was impaired, thereby inhibiting COX-2 expression. Furthermore, melatonin was found to synergistically enhance cisplatin-mediated

inhibition of AP-2β and hTERT expression. These results point to a sensitizing action of melatonin on cisplatin cytotoxicity through inactivation of NF-κB/COX-2 and AP-2β/hTERT signaling pathways in hepatocarcinoma cells[63].

Sorafenib is a multikinase inhibitor administered orally. This chemotherapeutic drug acts by inhibiting cell surface tyrosine kinase receptors. The drug blocks tumor cell proliferation and angiogenesis through inhibition of its main targets: platelet-derived growth factor receptor-beta receptor (PDGFRβ) and vascular endothelial growth factor receptor (VEGFR). The signaling pathways triggered by activation of these receptors are, as consequence, inhibited, being the main intracellular target Raf, a serine/threonine kinase. Sorafenib improves survival expectative and delays progression of HCC since it inhibits cell proliferation in a dose dependent manner.

The usefulness and safety of sorafenib in advanced HCC has been tested in different phase III randomized, double-blind, placebo-controlled trials. Although results obtained from these clinical studies have shown survival benefits, the efficacy of sorafenib treatment is frequently transient, and the activation of compensatory pathways in response to drug administration leads to tumor-acquired resistance, having a negative impact on the therapeutic outcome. Therefore, overcoming acquired resistance is required to potentiate cytotoxic effects of sorafenib, and the combination with other molecules could be a manageable approach to reduce adaptive response of tumor cells to the action of the drug.

Susceptibility to sorafenib in the presence of melatonin was assessed in HCC cell lines HepG2, HuH7, and Hep3B. When melatonin and sorafenib were applied together, they had a synergistic cytotoxic effect on HepG2 and HuH7 cells. Importantly, melatonin induced in Hep3B cell a susceptibility to doses of sorafenib that had no effect when administrated alone. Co-administration of sorafenib and melatonin triggered apoptosis in Hep3B cells, inducing a PARP hydrolysis and an increase in BAX expression. An early colocalization of mitochondria with lysosomes was also provoked, in parallel with an increase of the expression of mitophagy markers PINK1 and Parkin and a reduction of expression of mitofusin-2 and mtDNA compared with sorafenid administration alone. ROS production was enhanced and mitochondrial membrane depolarization is observed after combination of both compounds, suggesting a cooperative contribution to mitophagy induction. All these results demonstrate that the pro-oxidant capacity of melatonin and its actions altering mitochondria stability and turnover via mitophagy increase sensitivity in hepatocarcinoma cell lines might enhance the cytotoxic effect of sorafenib[64].

Very recently, (april, 2017) the last study to date about melatonin and sorafenib anticancer activity in hepatocellular carcinoma has been published. The results known up to now (some of them mentioned above) indicated that the pineal hormone has oncostatic actions on hepatocarcinoma through its anti-proliferative and pro-apoptotic actions. In this last recent report, both melatonin and sorafenib induced a dose-dependent growth inhibition of HuH-7 cells after two days of treatment, and the simultaneous administration of both substances enhanced the growth inhibition in a synergistic manner.

Colony formation assay indicated that co-treatment of HuH-7 cells with sorafenib and melatonin significantly dismissed the clonogenicity in comparison with those cells receiving only the chemotherapeutic drug. Other experimental approaches demonstrated that melatonin synergistically augmented the sorafenib-induced apoptosis, and this result is dependent on the activation of caspase-3 and the JNK/c-jun pathway, as demonstrated by the use of a specific inhibitor of the JNK/c-jun pathway (JNK inhibitor SP600125). This compound reversed the phosphorylation of c-jun, which in turn, impedes the activation of caspase-3 induced by co-treatment with melatonin and sorafenib in a dose-dependent manner. The main conclusion of this work is the confirmation that in combination with sorafenib synergistically inhibits proliferation and induces apoptosis at least in studies performed in human HCC cells. Therefore, inclusion of melatonin as an adjuvant to chemotherapy protocols including sorafenib could be a potential choice for advanced hepatocellular carcinoma[65].

Melatonin and lung cancer

Lung cancer (LC) is a rapidly increasing cause of cancer death among males and has surpassed breast cancer as the leading cause of cancer deceases in females. Despite improvements in survival for many other types of cancer in recent years, 5-year survival for LC has remained relatively poor, mainly because lung cancer is typically asymptomatic in its early stages of development, and even when symptoms appear, they are usually non-specific. Thus, most of LC patients are diagnosed after the disease has progressed to a more advanced stage. and treatment options are limited.

More than 85% of LC cases are currently classified as non-small cell lung cancer (NSCLC), for which the predicted 5-yr survival rate is lower than 16%. NSCLC is currently defined as one of the three histological phenotypes including adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. The latest data showed that the morbidity of lung adenocarcinoma occupied more than 50% in NSCLC, which requires much more attention. In the past decade, the two major treatments of lung adenocarcinoma have been surgery and chemoradiotherapy, which prolong the survival of patients with NSCLC. Nonetheless, the improvements are marginally modest, resulting in only 4% to 5% improvements in the 5-yr survival rates for stages I-III and prolongation of only months for stage IV. Thus, new advances including the discovery of oncogenic drivers and specific therapies for these drivers are urgently needed, specifically active endogenous, small molecules.

With respect to LC, melatonin has been shown to cause significant growth inhibition and apoptosis in NSCLC; however, the mechanisms responsible for the anti-cancer effects of melatonin in lung adenocarcinoma have not yet been fully elucidated. Some research has been designed to explore this anti-cancer activity of melatonin and the regulation of histone deacetylases (HDACs) after treatment with a pharmacological concentration of melatonin. These enzymes are critical regulators of gene expression removing the acetyl group from histones. Generally, they promote the condensation of chromatin and gene repression, particularly in oncogenesis, including p53, c-Myc, NF-κB, HIF-1α, HSP90, and others, apart from having a critical role in modulating the balance between pro- and anti-apoptotic proteins. Out of four groups, a common finding showed that class I HDACs are frequently overexpressed in various human cancers, especially in lung cancer, in which HDAC overexpression correlates with drug resistance and poor prognosis.

Related with this, Chongxi Fan et al. investigated the effect of melatonin on drug-induced cellular apoptosis against the cultured human lung adenocarcinoma cells and explored the role of HDAC signaling in this process[66]. Human lung adenocarcinoma cells (A549 and PC9) were treated twice with melatonin over a 12-h period for 24 hr. The results showed that melatonin, at concentrations of 0.9, 1.2, 1.5 mM, effectively suppressed human lung adenocarcinoma A549 and PC9 cell viability in a dose- and time-dependent manner, as well as cell adhesion and cell migration.

Their results indicate that melatonin treatment down-regulated HDAC1 expression and induced histone H3 acetylation in both A549 and PC9 cells. Moreover, knockdown of HDAC1 via siRNA markedly augmented melatonin-induced cell death. Additionally, the

anti-cancer effect of melatonin was synergistically improved when combined with the HDAC inhibitor, SAHA, showing an enhanced inhibition of HDAC1. In this study, the melatonin treatment significantly increased the cell apoptotic index in A549 and PC9 cells and it resulted in the down-regulation of Bcl2 expression and up-regulation of PUMA and Bax expression (all are key regulators of apoptosis and are balancing factors for the cell apoptotic program). Moreover, the apoptotic effects of melatonin were associated with rapid increases in intracellular ROS in both A549 and PC9 cells. Furthermore, the inhibition of HDAC1 by siRNA or SAHA enhanced melatonin-induced ROS production. Similarly, melatonin-induced ROS generation was associated with a significant consumption of intracellular GSH (Figure 5). These results indicated that the induction of the oxidative stress might be an important mechanism by which melatonin induces cell death in human lung adenocarcinoma cells. Finally, melatonin treatment downregulated PCNA expression in both A549 and PC9 cells, which is a molecular marker for proliferation because of its role in replication.

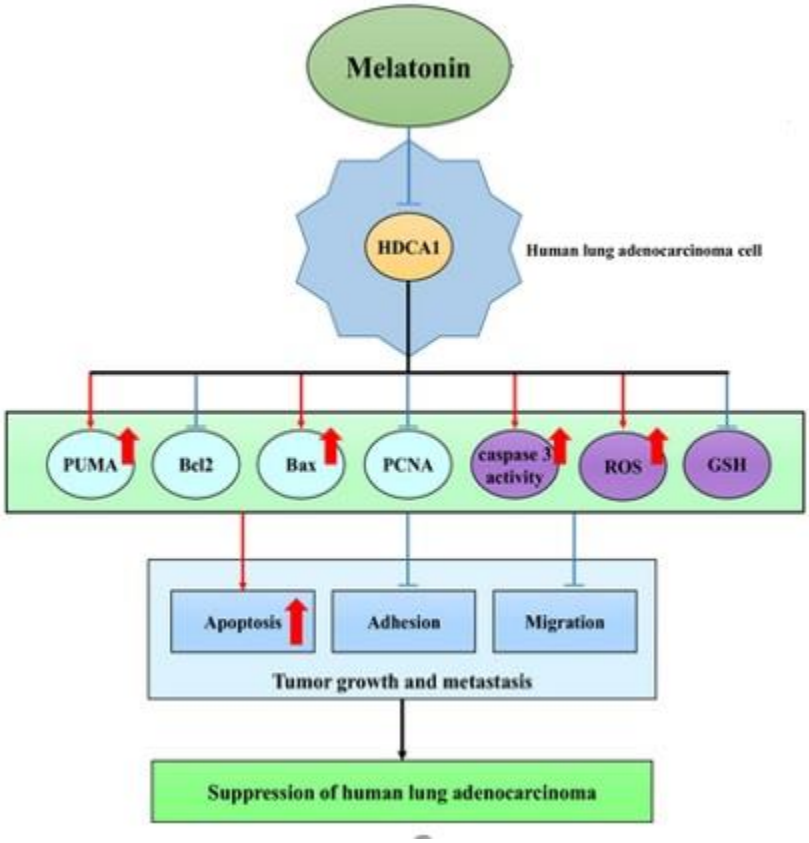

Figure 5: HDAC1 inhibition by melatonin leads to suppression of lung adenocarcinoma cells via induction of oxidative stress and activation of apoptotic pathways (Fan, C. et al. Journal of pineal research 2015)

In conclusion, melatonin treatment inhibits lung adenocarcinoma cell growth and metastasis via down-regulation of HDAC1 signaling. The downregulation of HDAC1 sensitizes human lung adenocarcinoma cells to melatonin treatment. Additionally, this potentiation of anti-cancer effects may be related to the up-regulation of pro-apoptotic pathways and activation of oxidative stress. Therefore, melatonin has multiple advantages that make it a strong candidate for therapeutic applications in lung adenocarcinoma. Additionally, the use of melatonin in combination with other known

HDAC inhibitors may be more effective for the treatment of lung cancer and should be examined further.

In 2014, Qiaoyun Zhou, Shuyu Gui et al, explored the effects of melatonin on the migration of human lung adenocarcinoma A549 cells and its mechanism. After A549 cells were treated with melatonin, the viability and migration of the cells were inhibited significantly. The relative migration rate of A549 cells treated with melatonin was only about 20% at 24 h [67].

Studies have demonstrated that many proteins, such as myosin light-chain kinase (MLCK) or osteopontin (OPN), play a critical role in non-muscle cell protrusion, contraction, and migration. Occludin is the main functional regulatory protein in Tight Junction (TJ) and is thought to be the most sensitive and reliable sign of TJ structure. They all involve JNK/MAPK pathway.

The expression level of OPN, MLCK and phosphorylation of MLC of A549 cells were reduced, while the expression of occludin was conversely elevated, and occludin located on the cell surface was obviously increased, which have the effect of inhibiting A549 cell metastasis. The phosphorylation status of JNK in A549 cells was also reduced when cells were treated by melatonin.

P. Plaimee et al. investigated melatonin's induction of apoptosis though biomolecular changes (lipid, protein, and nucleic acid/DNA) in the SK-LU-1 human NSCLC cell line[68]. The study was designed to test single measure FTIR microspectroscopy as a technique to track overall biochemical changes during cell death. They demonstrated anti-lung cancer activity of melatonin in a dose-dependent manner. SK-LU-1 cell growth was inhibited by 1 and 2 mM of melatonin. Meanwhile, cytotoxic effect of melatonin ranged between doses 2.5 and 10 mM.

Melatonin increased lipid content and reduced intensity of nucleic acid/DNA. Secondary protein structure at 1656 cm-1 (α-helix) was reduced and peak position of β-sheet structure (1637 cm-1) was shifted to lower frequency. Alteration in apoptotic proteins was demonstrated via caspase-3/7 activity induction.

Many specific markers can be observed during apoptosis, from cell changes such as membrane fluidity, ionic charge, membrane proteins and lipid structure. A well-known marker of apoptosis is the presence of phosphatidylserine on the outer leaflet of plasma membranes. Accordingly, the increased of peak FTIR spectra – between 3000 cm^{-1} and 2800 cm^{-1} from the plasma membrane phosphatidylserine – is called the 'marker band of the apoptosis'. Relative integral area of β-sheet and α-helix of melatonin-treated SK-LU-1 cells were respectively elevated and reduced compared to untreated cells.

Furthermore, caspases-3/7 – proteolytic enzymes involved in apoptotic activity were increased in melatonin-treated SK-LU-1 cells. Accordingly, their observation of shifting peak position of β-sheets confirms the conformation change of caspases, and increasing β-sheet peaks seems to confirm the induction of apoptosis through caspases activity. Nucleic acid content of SK-LU-1 cells was shown to be reduced by melatonin treatment.

In summary, they found that melatonin exerted anti-cancer activity on lung cancer cells by direct cytotoxicity as well as by induction of apoptosis by caspase activation. These were investigated by observing changes in the biomolecular structure of lipids, nucleic acids and proteins, by FTIR microspectroscopy. FTIR spectra revealed reduction in nucleic acid and α-helix protein structures, augmentation of lipid content and change in conformation of β-sheet protein structure, that seemed attributable to the apoptotic process. This work introduces FTIR microspectroscopy as an alternative for observing overall biochemical changes during apoptosis, with a single measurement. The precise mechanism of anti-cancer action by melatonin requires further investigation before conducting trials on its use in cancer patients.

In other research, Plaimee et al. evaluated the melatonin immunomodulatory action against the lung cancer cell line SK-LU-1, in co-culture with human peripheral blood mononuclear cells (PBMC)[69]. For that, melatonin was tested on the SK-LU-1 cell line only after 24h incubation (direct effect), and on the co-culture system of SK-LU-1 and PBMC to investigate any indirect effect. As cisplatin is the first-line treatment for NSCLC in most countries, they used cisplatin-sensitive NSCLC grade III (SK-LU-1) in *in vitro* co-culture with human PBMC.

Their preliminary experiments demonstrated that melatonin added at high doses (1 mM) inhibited SK-LU-1 cell growth after 24 h incubation. Flow cytometry performed on the annexin V/PI-stained cells resulted in increase in frequency of number of apoptotic SK-LU-1 cells in co-culture with melatonin-activated PBMC. Moreover, pro-apoptotic effects of co-culture on SK-LU-1 cells was similar. On the other hand, it has been demonstrated that activated PBMC can synthesize and secrete endogenous melatonin, exerting intra-, auto- and paracrine immunomodulatory effects. Thus, enhanced SK-LU-1 cancer cell apoptosis in co-culture with PBMC might result from immunomodulatory effects of melatonin, of both exo- and endogenous origin.

Melatonin introduced to the co-culture caused further depletion of intracellular GSH in the SK-LU-1 cells with no effect on $O_2^{\bullet-}$ and H_2O_2, which can be interpreted as higher oxidative stress of target cells, due to action of melatonin. This depletion of GSH was concomitant with G_0/G_1 phase arrest induction in co-culture with melatonin-supplemented PBMC.

In summary, direct cytotoxic activity of melatonin at high doses, on lung cancer cell line SK-LU-1 was observed in culture. In contrast, an indirect effect was exhibited at lower doses, enhancing human PBMC to counteract proliferation of cancer cells. Increased apoptotic cell death, arrest of cell cycle phase and imbalance of oxidative status in the cancer cell lie were observed, as the immunomodulatory effect of melatonin in co-culture. These results imply that melatonin had an indirect effect on these lung cancer cells by enhancement of immunomodulatory activity.

The use of tyrosine kinase inhibitors (TKIs) to target active epidermal growth factor receptor (EGFR)-harbouring mutations has been effective in patients with advanced NSCLC. However, the use of TKIs in NSCLS patients with somatic EGFR mutations, particularly T790M, causes drug resistance. Yun et al. treated H1975 and HCC827 cells with melatonin in combination with gefitinib, and cell viability, cell cycle

progression, apoptosis, and EGFR, AKT, p38, Bcl-2, Bcl-xL, caspase 3 and Bad protein levels were examined[70].

Treatment with melatonin dose-dependently decreased the viability of H1975 cells harbouring the T790M somatic mutation compared to HCC827 cells with an EGFR active mutation. Melatonin-mediated cell death resulted in decreased phosphorylation of EGFR, which suggests the potential benefit of combination therapy with TKIs in patients with EGFR inhibitory mutations, and Akt, leading to attenuated expression of survival proteins, such as Bcl-2, Bcl-xL and survivin, and activated caspase 3 in H1975 cells, but not in HCC827 cells. However, they did not observe a significant change in expression of cell cycle proteins, such as cyclin D, cyclin A, p21 and CDK4 in H1975 cells. In other words, melatonin suppressed activation of Akt via inhibition of EGFR phosphorylation but did not affect MAPK signaling, indicating that simultaneous activation of multiple signaling pathways is required for MAPK induction.

Therefore, melatonin induces apoptosis and down-regulates survival related proteins such as Bcl-2 and Bad phosphorylation via inhibition of EGFR/Akt signaling. Melatonin also increases the sensitivity to gefitinib of H1975 cells containing active and somatic EGFR mutations, which leads to TKI resistance. These findings suggest that melatonin may be a potent chemotherapeutic agent in combination with gefitinib for treatment of NSCLC harbouring EGFR mutations resistant to TKI monotherapy.

Moreover, co-treatment of H1975 cells caused consistent down-regulation of EGFR phosphorylation and induced apoptosis compared to treatment with gefitinib or melatonin alone.

Leukemia and melatonin

Leukemia is a blood cancer which starts in blood-forming tissue, usually the bone marrow. Leukemia involves an over-production of white blood cells. There are many different types of leukemia, although they are classified in two main groups based on the type of blood stem cell they developed from. Lymphocytic leukemia involves over-production of lymphocytes, and myelogenous leukemia involves over-production of white blood cells called granulocytes. Over time, leukemia cells crowd out normal blood cells leading to serious bleeding and infection.

One of the first works relating melatonin and blood cancer was published 20 years ago, and its main goal was to address the potential protective effects of melatonin on bone marrow of rodents treated with cytotoxic drugs. When chemotherapeutic drugs are administered to leukemia (and other kinds of cancers) patients, one of the most serious problems is myelosuppression. When melatonin was administered with aracytin (cytarabine), an antimetabolic agent that inhibits both DNA and RNA polymerases and nucleotide reductase enzymes needed for DNA synthesis, the red blood cells count and the blood platelet count were significantly increased. Melatonin injection significantly increased total protein and globulin. These results indicate that melatonin protects bone marrow from the damage caused by chemotherapy, and stimulates the suppressed bone marrow[71].

Much more recently, in 2009, the effect of melatonin in combination with different drugs included in initial therapy protocols for patients with chronic lymphocytic leukemia (CLL) was tested. Cyclophosmamide (an alkylating agent), bromocriptine (a dopamine agonist), somatostatin and retinoids were administered in combination with melatonin to four patients. When these agents are given to stage I CLL patients, usually a complete response is achieved, but all patients relapse with a progression free survival not higher than 48 months. The four patients enrolled in this study did not have disease recurrence after 2, 6 and 10 years and showed no toxicity, pointing to melatonin as a promising agent to be included in CLL therapy protocols[72].

Studies in populations

A 2007 report by the International Agency for Research on Cancer classified night-shift work as possibly carcinogenic to humans. Circadian rhythms are endogenous and self-sustained oscillations of multiple biological processes with approximately 24-h rhythmicity. Circadian genes and their protein products constitute the molecular components of the circadian oscillator that form positive/negative feedback loops and generate circadian rhythms. The circadian regulation extends from core clock genes to various clock-controlled genes that include various cell cycle genes. Aberrant expression of circadian clock genes may lead to genomic instability and accelerated cellular proliferation and, ultimately, potentially promoting carcinogenesis.

There is a study addressing the simultaneous expression of four circadian clock genes (Bmal1, Clock, Per1 and Per2) and three clock-controlled cell cycle genes (Myc, Cyclin D1 and Wee1) at mRNA level and determination of serum melatonin levels in peripheral blood samples of 37 CLL, patients and equal number of age- and sex- matched healthy

controls. The circadian clock regulates not only the daily cycles but also modulates the immune response, and thus, it may contribute to the lymphoid neoplasms etiology. The aberrant expression of circadian clock genes can lead to aberrant expression of their downstream targets that are involved in cell proliferation and apoptosis and hence may result in manifestation of CLL.

Shift-work and low melatonin levels may also contribute in etiology of CLL by further perturbing of circadian clock. The results of this study showed that expression of Bmal1, Per1, Per2 and Wee1 was significantly down-regulated whereas Myc and Cyclin D1 expression was up-regulated in CLL patients. When expression of these genes was compared between shift-workers and non-shift-workers within the CLL group, the expression was found to be more aberrant in shift-workers, although only Myc and Cyclin D1 differences were significant. Interestingly, serum melatonin levels were significantly lower in CLL subjects as compares to healthy controls whereas melatonin levels were found still lower in shift-workers as compared to non-shift-workers within the CLL group[73].

Another work was performed to investigate an association between night shift work and CLL. The size of the groups enrolled in the study were 321 incident CLL cases and 1728 population-based control in five areas of Spain. Overall, working in night shifts was not associated with CLL. However, long-term night shift (>20 years) was positively associated with CLL, although no linear trend was observed. A positive association was observed with years of rotating shift work[74].

Melatonin and chemotherapy in leukemia. Molecular pathways involved

In the recent years, the molecular mechanisms by which melatonin exerts its anti-cancer effects in leukemia cells are starting to be clarified. In Molt-3 cell line, a malignant human leukemia lymphoid cell line that is especially sensitive to the cytotoxic action of natural compounds and displays functional, both the intrinsic and the extrinsic, apoptotic pathways, melatonin efficiently reduced the number of Molt-3 cells in a concentration and time-dependent manner. The administration of the indoleamine augmented the percentage of cells with a hypodiploid DNA content, increased the number of cells staining positive for annexin V and provoked changes indicative of apoptotic cell death. Inhibition of caspases revealed that melatonin also activates in parallel an alternative caspase-independent cell death modality. The intrinsic apoptotic pathway seems to play a key role in melatonin-induced cell death because caspase-9, but not caspase 8, was activated in this cell line. Thus, activation of caspase-9 was an early event which is associated with an increase in the ratio Bax/Bcl-2 and with an elevation in cytochrome c in the cytosol.

Melatonin also induced ROS formation, prior to caspase activation, in a time- and concentration-dependent manner. The pineal hormone stimulated an acute and persistent generation of ROS, probably through a receptor-independent mechanism because the effect was only detected with supra-physiological concentrations. Whether the quick ROS production stimulated by melatonin in Molt-3 cells displays any effect on cell metabolism remains to be elucidated. In conclusion, the apoptotic mechanisms

involved seemed to be caspase-dependent but ROS-independent, involving outer mitochondrial membrane permeabilization and cytochrome c release[75].

The effects of melatonin in a panel of cell lines representing different lymphoid malignancies were addressed. Four cell lines were included in the study: Ramos (Epstein-Barr virus-negative BL), SU-DHL-4 (diffuse large B cell lymphoma), DoHH2 (follicular B non-Hodgkin lymphoma) and JURKAT (acute T cell leukemia). Melatonin promoted cell cycle arrest and apoptosis in all these cells, although there was marked variations in responses among different cell lines (sensitivity: Ramos similar to DoHH2 > SU-DHL-4 > JURKAT).

The pineal hormone triggered apoptosis relatively rapid, with increased caspase 3 and PARP cleavage detected just 30 minute after melatonin addition. Additionally, there was evidence for rapid processing of caspase 9, in parallel with a breakdown of the mitochondrial inner transmembrane potential. In contrast, caspase activation was obtained only in SU-DHL-4 and Ramos cells following melatonin treatment suggesting that the extrinsic pathway is not a common feature in melatonin-induced apoptosis in malignant lymphocytes. All the cell lines enrolled in this study express the high-affinity melatonin receptors, MT1 and MT2. However, melatonin-induced caspase activation appeared to be independent of these receptors[76].

Not all the reports concerning the use of melatonin in combination with chemotherapeutic drugs found positive effects. The effect of melatonin on the cytotoxicity of Ara-C, ETO and DNR was tested in Jurkat, MOLT-4, HL-60, Daudi, K562 and CMK leukemia cells in *vitro*. These compounds are the commonly used agents in treatment of childhood leukemia. A pharmacological concentration of melatonin in Jurkat, CMK and MOLT-4 cells had a cytotoxic effect, associated with the generation of reactive oxygen species. Although melatonin itself has some cytotoxic effects on the tumor cell lines studied, this indoleamine did not enhance the cytotoxicity of the drugs tested in leukemia cells. None of the chemotherapy compounds used, Ara-C, ETO and DNR, exerted enhanced cytotoxicity when co-incubated with 10^{-5} M and 10^{-3} M concentrations of melatonin in Jurkat, MOLT-4, HL-60, Daudi, K562 and CMK cells[77].

Most promising effects were found when melatonin was combined with puromycin in acute pro-myelocytic leukemia HL-60 cells. Puromycin is an amino nucleoside antibiotic acting in the ribosome, causing premature chain termination during translation. Melatonin did not have better cytotoxic effects than puromycin. However, when added together, melatonin significantly augmented the cytotoxic effects of the antibiotic. The molecular mechanisms have been characterized. Melatonin by itself was able to suppress phospho-Chk1 levels, while activating phospho-Chk2 in HL-60 cells. When added in combination with puromycin, the pineal hormone had a synergistic effect with the antibiotic on cytotoxicity. The signaling pathways triggered involve caspase activation, PARP cleavage and AMPKα activation. Additionally, melatonin suppressed the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-x_L in puromycin-treated HL-60 cells. Furthermore, melatonin enhanced puromycin-induced apoptosis and suppressed G2/M arrest in HL-60 cells[78].

Acquired drug resistance remains a serious obstacle in leukemia therapy. In many cases, other leukemias are intrinsically drug resistant. After the characterization of the human MDR1 gene, which encodes for a pump that efflux chemicals out of the cells (P-glycoprotein), it has been demonstrated that the levels of MDR1 RNAs are elevated in leukemia and many other kinds of cancers. Other export pump that is usually upregulated in multidrug resistance is ATP-binding cassette subfamily G member 2 (ABCG2).

Clofarabine is one of the newly developed nucleoside antimetabolites characterized for a broad cytotoxic activity, with high expectations of therapeutic efficacy. Clofarabine is currently approved for relapsed acute lymphoblastic leukemia. A study showed that the development of clofarabine resistance in two newly established clofarabine-resistant acute lymphoblastic leukemia cell lines was accompanied by down-regulation of a key gene in clofarabine metabolism, deoxycytidine kinase (dCK). This down-regulation was due to gene promoter hypoacetylation.

Incubation with melatonin resulted in a marked increase in the cytotoxicity of clofarabine in leukemic resistant cells. Melatonin treatment induced higher levels of acetylation, which suggest that melatonin alters DNA accessibility via histone acetylation and relaxation of the chromatin structure, which could allow clofarabine to target the DNA more efficiently.

The molecular mechanisms of clofarabine resistance probably include an ABCG2 overexpression simultaneous to a decreased expression of dCK gene with methylated promoter regions. No changes in ABCG2 expression nor any changes in methylation status in clofarabine-resistant cells where identified. However, acetylation of histone increases gene expression, which is known to be an epigenetic mechanism. This is the first report to find that histone deacetylation of the dCK promoter is responsible for the decreased expression of dCK in clofarabine-resistant leukemic cells. It was also found that histone deacetylation status differs between NALM6 cells, which are derived from B-cell leukemia, and SKW3 cells, which are derived from T-cell leukemia.[79]

The most recent works aim to clarify the relationship among anti-cancer compounds of new generation, melatonin and reactive oxygen species for induction of apoptosis. A report published in 2017 analyzed several parameters in leukemia cells and normal lymphocytes: cell viability, induction of apoptosis, level of reactive oxygen species and levels of protein-carbonyl products. The study covered for 11 new-generation newly developed chemotherapeutical drugs and melatonin. The most promising results of this work point to melatonin as a molecule able to confer synergistic cytotoxicity with most of the drugs tested. The most impressive results were accomplished with combinations of everolimus plus melatonin or else barasertib plus melatonin, where a marked synergistic effect on cytotoxicity was accompanied by a strong induction of apoptosis. Very remarkably, these combinations of drugs did not affect the viability of normal lymphocytes. Apoptosis was correlated with a decrease of ROS to a level below that of control untreated cells. These recent data suggest that melatonin might be an effective supplementary component in chemotherapy of leukemias, allowing the therapeutic doses to be reduced and minimizing the undesirable chemotherapy side effects[80].

Melatonin, glioblastoma and glioma

Glioblastoma, also known as glioblastoma multiforme (GBM), is the most common primary brain tumor. Glioblastomas represent 15% of brain tumors. They can either start from normal brain cells or develop from an already existing low-grade astrocytoma. Glioblastoma is very aggressive and it is also one of the most lethal cancers, with an average life expectancy lower than 1 year. The most common length of survival following diagnosis is 12 to 15 months with less than 3% to 5% of people surviving longer than five years. Without treatment survival is typically 3 months. It is the most common cancer that begins within the brain and the second most common brain tumor after meningioma. About 3 per 100,000 people develop the disease a year. It most often begins in the fifth or sixth decade of life and occurs more commonly in males than females.

Signs and symptoms of GBM are initially non-specific. The most common manifestations are usually a consequence of the increase pressure in the brain. Among them, patients frequently suffer from headache, nausea and drowsiness. Depending on the location of the tumor, patients can undergo many other symptoms such as weakness on one of the sides of the body, memory and/or speech impairments, and visual problems. GBMs will usually develop in the hemispheres of the brain, but they can be also located in the spinal cord. They arise from astrocytes, one of the cell types that make up the supportive tissue of the brain, although they usually contain a mix of cell types. They receive a rich blood supply due to the abundance of blood vessels.

There is no clear way to prevent the disease. Treatment of GBM patients is extremely challenging, as complete surgical resection of the tumor is very difficult and glioblastomas are refractory to current chemotherapy regimens. Typically, treatment involves surgery followed by either chemotherapy or radiation. Temozolomide is frequently employed as part of chemotherapy protocols. High dose steroids may be used to help reduce swelling and palliate the symptoms. Despite treatments, the cancer usually recurs. Immunotherapy is being studied in glioblastoma with promising results.

As one of the deadliest cancers, GBM often diffusely invades into adjacent normal brain tissue and migrates a considerable distance from the primary tumor area. In fact, the infiltrative growth into the surrounding normal brain parenchyma makes it impossible to achieve complete surgical resection without causing severe neurologic damage and thus contributes to the poor prognosis of this type of tumor. Understanding the mechanisms of GBM invasion and migration is therefore vital for designing effective therapeutic interventions to prevent the spread of this disease. Growing evidence suggests that the tumor microenvironment is as important as the intrinsic properties of tumor cells in determining tumor progression and patient prognosis.

There are not many studies addressing the role of melatonin at molecular level on this type of tumor. One of them has been performed using as a model the U251 and U87 glioblastoma cell lines[81]. There is growing evidence that hypoxia is a major driving factor to direct GBM toward a more aggressive and malignant state. Hypoxia stimulates U251 and U87 glioblastoma cells migration and invasion. Overexpression of the HIF-1α protein is often detected in GBM, and it is an essential component in glioblastoma cell's migratory and invasive activities under hypoxic conditions. Melatonin blocks HIF-1α and

its transcriptional products MMP-2 and VEGF, leading to a marked inhibition of migration and invasion of both U251 and U87 glioblastoma cell lines in response to hypoxia.

It seems that melatonin treatment results in a destabilization of the hypoxia induced HIF-1α factor. Melatonin likely exerts this effect via its antioxidant activity against ROS synthesized in the glioblastoma cells in response to hypoxia. Interestingly, HIF-1α silencing by using small interfering RNAs inhibited both cell migration and invasion, effect that seems to be associated with MMP-2 and VEGF, two of the factors that are transcribed in response to HIF-1α. The main conclusion of this work is that melatonin effectively blocks migration and invasion of glioblastoma cells, and the molecular pathway involved in this inhibitory action of the pineal hormone is suppression of HIF-1α mediated transcriptional activity.

Another report addressing the effect of melatonin on glioblastoma has been performed using Glioblastoma stem-like cells (GSCs) isolated from surgical specimens from patients with GBM[82]. This type of brain tumor contains cellular hierarchies harboring a subpopulation of stem-like cells that grow as spheres with self-renewing and tumor-propagating capacity. Therefore, it is possible to efficiently propagate tumors in xenograft models intracranially implanted in nude mice. Numerous studies previously reported that these GSCs play a particularly important role in maintaining tumor growth, therapeutic resistance, and tumor recurrence.

Histone modifications represent a major epigenetic mechanism, which deregulation can trigger tumor initiation and propagation. The EZH2 subunit of the Enhancer Of zeste homolog 2/polycomb repressive complex 2 (PRC2/EZH2) has been characterized as a major inhibitor of the expression of tumor suppressors, including p19, Bim, p57, E-cadherin, and RUNX3, and additionally, it may be critical in maintaining the self-renewal capacity of normal or cancer stem cells by repressing lineage-specific differentiation programs (Figure 6*)*. Furthermore, recent investigations revealed that EZH2 exhibits two separated functions: transcriptional inhibition through histone methylation and signal transducer and activator of transcription 3 (STAT3) induction, with both functions actively involved in GSC self-renewal and GBM malignancy.

EZH2 overexpression was reported to occur in GBM, and its expression level directly correlates and serves as a positive-predictive marker for the capability of the tumor to migrate and to invade adjacent tissues. PRC2/EZH2-mediated transcriptional silencing plays a critical role in the maintenance of stem cell status in malignant stem cells.

Melatonin directly targets the glioblastoma tumor cells by altering the glioblastoma stem-like cells and inhibiting their proliferation. In addition to melatonin-induced decreases in GSC self-renewal and clonogenic capability, melatonin inhibited EZH2 S21 phosphorylation, EZH2-STAT3 interaction and altered histone modifications in GSCs. The inhibitory effect of melatonin on GSC growth mainly results from alterations in EZH2 activity. Remarkably, AKT1 overexpression reversed this effect, providing further evidence that AKT is a key downstream effector of melatonin in GSCs. The results of this work suggest that melatonin may have inhibitory effects on glioblastoma migration and invasion, by attenuating multiple key signaling pathways involved in GSCs self-renewal

and survival, supporting the idea of melatonin as a promising glioblastoma therapeutic agent.

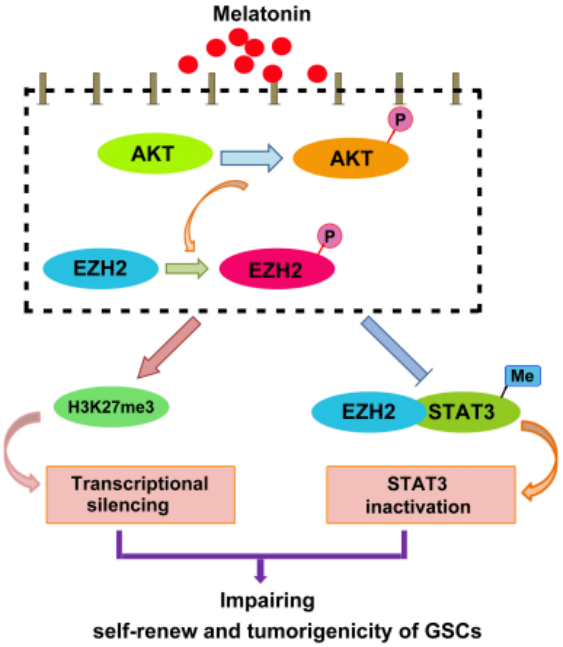

Figure 6: Melatonin inhibits tumorigenicity of glioblastoma stem-like cells via the AKT–EZH2–STAT3 signaling axis (Chen, X. et a., Journal of pineal research 2016l)

Very recently, another report has related the potential tumor inhibitor actions of melatonin on glioblastoma with the EZH2-NOTCH1 pathways[83]. The Notch signaling pathway is important for cell to cell communication. NOTCH receptors and ligands are single-pass transmembrane proteins that play an important role in cell fate decisions during embryonic and postnatal including neuronal function and development. NOTCH receptors are overexpressed in many kinds of cancers. Concretely, NOTCH1 has been identified as the key signal molecule that regulates the EZH2-mediated effects of melatonin in the GSCs. EZH2 directly regulates NOTCH1 expression by directly binding to the NOTCH1 promoter.

Melatonin inhibited GSCs viability and self-renewal accompanied by a decrease in the expression of stem cell markers, as well as, a reduction in the expression levels of EZH2 and NOTCH1 signaling pathway components. NOTCH1 was one of the most significantly down-regulated genes. Coincidentally, melatonin remarkably reduced NOTCH1 in the GSCs as well as other NOTCH1 signaling pathway components (e.g. CCND1, CCNE2 and HES1), which are regulated by NOTCH1. The active NOTCH1 protein segment, NOTCH intracellular domain 1 (NICD1) was also deregulated, suggesting that NOTCH1 might mediate the effects of EZH2 upon treatment of melatonin. Collectively, melatonin seems to perform its function partly by suppressing GSC properties through EZH2-NOTCH1 signaling axis.

Gliomas are tumors generally occurring in the brain or spine, and develops from glial cells. They represent about 80% of all malignant brain tumors. Gliomas could develop from astrocytes, oligodendrocytes or ependymal cells. These tumors can be classified and low grade or benign glioma and high grade or malignant glioma. About 30% of

gliomas are malignant. Gliomas commonly progress more slowly than glioblastomas, although their prognosis is not very good. There is no cure and treatments are focused to slow down its growth and to manage symptoms. The average survival once diagnosed is on an average of one year, although the life expectancy varies depending on whether the tumor is benign or malignant.

The infiltrative nature of gliomas, with their propensity for microscopic dissemination through the brain and spinal cord, makes it very difficult to achieve a complete resection. As a result, "total resection" invariably fails to completely remove all microscopic cancerous focus. An additional serious problem is that complete elimination of the tumor usually compromises performance and quality of life for patients. In general, microsurgical resection is performed for high grade gliomas, prior to concomitant chemotherapy and radiation. Achieving at least 70-80% resection provides a significate improvement in survival benefits.

Temozolomide is a new drug that has shown promising results when used in malignant gliomas. This chemotherapeutic agent is a second-generation alkylating agent able to enter the cerebrospinal fluid and do not require hepatic metabolism for activation. Some preclinical studies have evaluated the combination of temozolomide with an inhibitor of the DNA repair protein O6-alkylguanine alkyl transferase. Temozolomide showed effectiveness to overcome resistance to chemotherapy in malignant glioma.

It has been demonstrated that melatonin is able to inhibit the growth of glioma cells, based on its antioxidant properties. Most of the studies assessing the effects of melatonin on glioma have been performed in the C6 glioma cell line. Melatonin, at pharmacological doses, impairs the proliferation of C6 cells and reduces the local biosynthesis of estrogens. Melatonin counteracts the stimulatory effects of testosterone on proliferation, fact that might be explained since melatonin inhibits both the expression and activity or aromatase, the enzyme that converts androgens into estrogens[84].

Melatonin decreases cell proliferation of glioma cells both in *vivo* and in *vitro*. Melatonin reduces brain tumor stem cells (BTSCs) proliferation and diminishes the self-renewal and clonogenic ability, all accompanied by a reduction in the expression of stem cell markers. Recent evidence indicates that BTSCs overexpress members of adenosine triphosphate binding cassette (ABC) transporters, proteins responsible for the efflux of chemotherapeutic drugs in cancer cells. Overexpression of ABC transporters correlates with multidrug resistance and tumor relapse.

Combinations of melatonin and temozolomide, the current drug administered to malignant glioma patients, have a synergistic toxic effect both in BTSCs and A172, a malignant glioma cell line. Importantly, this cytotoxic effect seems to be specific for cancer-derived stem cells as no induction of cell death has been observed in normal human neural stem cells.

When melatonin actions were analyzed at the molecular level, it was found that the pineal hormone increased the methylation levels of the ATP-binding cassette sub-family G member 2 (ABCG2) promoter, a member of the ABC transporters. These methylation

results in an inhibition of ABCG2 expression and consequently, a loss of its function. The observed decrease in expression and function of ABCG2/BCRP after melatonin treatment in both BTSCs and glioblastoma cell lines could be responsible for the potentiation of chemotherapeutic drug cytotoxicity. It is well known that some chemotherapeutic drugs such as doxorubicin are substrates for the ABCG2/BCRP transporter, which actively exports drugs from the intracellular to the extracellular space. Recently, it has been proposed that temozolomide might be a substrate for the ABCG2/BCRP transporter. Thus, melatonin might exert its action on glioma cells by inhibiting the ABC transporters, sensitizing them to drugs such as temozolomide[85].

Conclusions and remarks

The number of published articles supporting the oncostatic role of melatonin on experimental models of a variety of cancers is growing exponentially during the last years. Melatonin is a substance with multiple physiological effects playing important roles in different processes, including circadian rhythm, sleep and reproduction.

It is noticeable the multiple mechanisms that have been described as involved in melatonin mediated inhibition of cancer genesis, progression and metastasis. Initially, most of the studies focused on estrogen responsive breast cancer models. Many studies performed in animal models and estrogen responsive cell lines have pointed to melatonin as a molecule able to inhibit the proliferation and the metastatic behaviour. These results have been obtained both *in vitro* in human breast cancer derived cell lines and i*n vivo,* in mammary cancer models established in rodents, where the pineal hormone diminishes the incidence of mammary tumors and limits their growth.

In this kind of cancer, it seems that the main mechanism by which the pineal hormone reduces proliferation and progression of estrogen-positive mammary tumors is by interfering with estrogen signaling pathways. Many hypotheses have been proposed to explain the oncostatic role of melatonin: i) Through an indirect mechanism, by downregulating the synthesis of estrogens via inhibition of the hypothalamic-pituitary-reproductive axis. ii) Through a direct mechanism, by interfering with the activation of cytoplasmic estradiol receptors expressed in the cancer cell, thus behaving as a selective estrogen receptor modulator (SERM). iii) By down-regulating the enzymes necessary for the synthesis of estrogens in other tissues, therefore behaving as a selective estrogen enzyme modulator (SEEM).

In breast cancer models, it has been recently demonstrated that melatonin modulates the paracrine signals inter-communicating malignant epithelial cancer cells and proximal tissues, such as surrounding adipose tissue (fibroblasts and adipocytes) and endothelial cells. The main action of melatonin takes account through the downregulation of the levels of growth factors and cytokines released by breast tumor cells (cytokine gene activation increases these molecules´s concentrations in the proximity of the tumor promoting cellular proliferation and tumor progression). Therefore, the pineal hormone seems to have an antiangiogenic role.

In the past few years, a great amount of evidence describing the effects of melatonin mitigating cancer at the initial stages, progression and metastasis have been documented. These inhibitory effects of melatonin have been reported not only for estrogen-dependent breast tumors, but also for numerous different cancers, including gastric cancer, ovarian carcinoma, pancreatic ductal carcinoma, leukemic cell lines, cervical cancer and non-small lung carcinoma. Most of the results are positive, and melatonin has been described as an inhibitor of tumor growth under both *in vitro* and *in vivo* experimental conditions. Several actions of melatonin have been proposed to explain the mechanisms by which melatonin achieves its anti-cancer effects: modulation of signaling transduction pathways triggered by its binding to the MT-1 and MT-2 cell membrane receptors, anti-oxidant actions, anti-angiogenesis, anti-inflammatory effects, reduction on growth factors uptake, inhibition of ATP-dependent drug efflux

pumps involved in chemotherapy resistance and inhibition of telomerase (see appendixes 2 and 3).

Such variety of mechanisms proposed suggest that there must be a common denominator for all these actions still not discovered. Multiple actions of melatonin are equally as common in normal cells and cancerous tissues, supporting the idea that the primary action of melatonin remains unidentified and what the different groups of research described are epiphenomena of melatonin´s fundamental function.

It is becoming clear that melatonin co-administration improves the sensitivity of cancers to inhibition by conventional chemotherapeutic agents. One of the most striking recent findings described, concerning melatonin´s anti-cancer activity is its ability to transform cancers that are totally resistant to chemotherapeutic drugs to a sensitive to chemotherapy state. Melatonin also seems to inhibit molecular pathways implicated in metastasis by limiting the entrance of cancer cells into the vascular system (Figure 7). Despite all these findings, the underlying mechanisms by which melatonin exerts its effects, again, are not well identified.

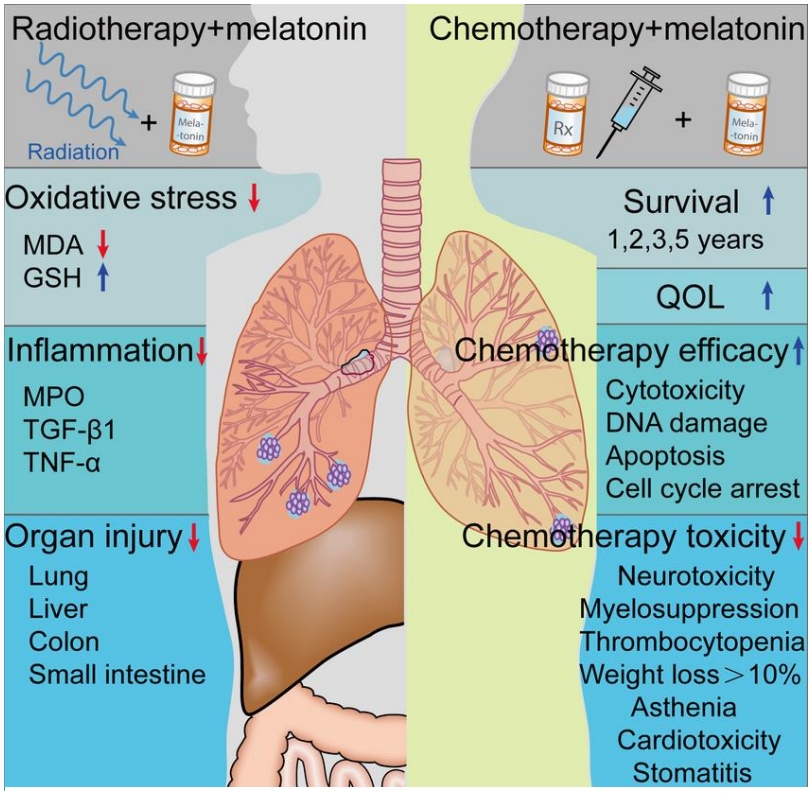

Figure 7: Effect of melatonin on radio- or chemotherapy (Zhiqiang Ma et al., Oncotarget, 2016)

The results arisen in the past few years also suggest that melatonin, either used alone or along with chemotherapeutic agents in cancer patients diagnosed with advanced solid tumors, contributed to ameliorate the outcomes of cancer regression and life expectancy of the patients receiving the pineal hormone. It has been reported that chemotherapeutic treatments were generally better tolerated by those patients who were simultaneously receiving melatonin, and the long-term side effects were palliated.

After the first and pioneer clinical study of melatonin potential positive effects in untreatable advanced cancer patients performed by Lissoni et al. in Italy, some other studies have been published. The collateral toxicity and potential lethality of radiotherapy and chemotherapeutic agents is a major problem concerning their use. It is a common fact that normal tissues are seriously damaged by these treatments. Melatonin, in many experimental models has been shown to mitigate acute and chronic cell damage, such as cardiac damage, hepatic and renal toxicity. Despite findings as these, the administration of melatonin to patients who could potentially benefit from such co-treatment is not yet been applied.

To date, the main criticisms concerning the positive actions of melatonin is the limited number of patients enrolled in the published clinical trials performed to date. It seems obvious that further studies are required, including additional randomized double-blind controlled trials with much larger sample sizes and implicating several international hospital centres, since the data available nowadays arise from clinical trials including only a few hundred patients.

In addition, it is necessary to say that not all the studies performed to date confirmed that melatonin is a molecule that improves life expectancy and ameliorates the adverse effects of chemotherapy. For example, a clinical trial involving patients suffering from advanced lung or gastrointestinal cancer, showed that melatonin did not exhibit any beneficial effect, and as consequence, the value of melatonin as an adjuvant in the treatment of cancer remains unclear from these data.

One of the main objectives of the present review was to summarize the current knowledge regarding the interplay of melatonin and chemotherapy in other cancer models different from estrogen responsive mammary tumors. Changes in gene expression after treatment with melatonin alone or in combination with chemotherapy remain largely unknown. Apart from tamoxifen, there is limited information from research performed at the molecular level addressing the potential benefits of co-treatment of melatonin with chemotherapeutic agents, although there have been many published studies proving that melatonin defers the progression of experimental tumors both *in vivo* and *in vitro* models. Many kinds of cancers have been studied and multiple mechanisms have been proposed as involved in the oncostatic actions of the pineal hormone.

The pineal hormone is an endogenous produced substance with a high potential of being included as an effective anticancer molecule in the prevention and treatment of, not only hormone-dependent cancers, but also, other types of cancer, since its inhibitory effects have been demonstrated in gastric, lung, pancreatic and hematopoietic cancers. We have to take in mind that melatonin is an endogenously produced molecule lacking any dangerous toxicity or negative side effects at any dose.

In the next future, additional research must be conducted to clarify if melatonin administration in combination with chemotherapeutic agents may constitute a novel anti-cancer treatment. In particular, future research addressing the role of melatonin as a non-toxic and low-cost drug to be considered in breast and other types of tumors must be conducted, particularly at the molecular level. Systematic screenings addressing the

effects of chemotherapy on genes known to be altered in different types of cancer, and on how melatonin can modulate the expression and activity of those genes, either when acting alone or in combination with chemotherapy, should be performed.

Once larger clinical trials and additional molecular studies (including gene expression profiles, post-translational modifications and individual gene tests) have been conducted, it may be reasonable to recommend melatonin as a potential drug to be considered in the treatment of breast cancer. There are many individuals suffering from many types of cancer who could potentially benefit from such co-treatments.

Acknowledgements

Firstly, I would like to thank the University of Cantabria, teachers, tutors and colleagues in general, who have mentored and guided me, over the past years in my learning experience that I have valued so much.

Lastly, I would like to give special mention and my deepest gratitude to my tutor Carlos Manuel Martínez Campa, my General Physiology teacher, for his generosity in which he has dedicated his attention and his time, to gather the information on the subject matter. For his extraordinary and exhaustive work of bibliographic selection, documentation and images that support this work.

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APPENDIX 1: ABBREVIATIONS

13-HODE: 13-hydroxyoctadecadienoic acid

17β-HSD1: 17β-hydroxysteroid dehydrogenase type 1

4E-BP1: 4E-binding protein 1

AANAT: Aryl-alkyl-

amine N-acetyltransferase

ABC: ATP-binding cassette

ABCG2: ATP-binding cassette subfamily G member 2

AFMK: N1-acetyl-N2formyl-5-methoxykynuramine

AMK: N1-acetyl5-methoxykynuramine

AP1: Activator protein 1

AR: Androgen receptor

ASMT: Acetylserotonin O-methyltransferase

ATF6: Activating Transcription Factor 6

BTSCs: Brain stem cells

c3OHM: Cyclic-3-hydroxymelatonin

cAMP: Cyclic adenosine monophosphate

CAT: Catalase

CDK: Cyclin-dependent kinase

COX-2: Cyclooxygenase-2

cGMP: Cyclic Guanosine Monophosphate

CLL: Chronic lymphocytic leukemia

CNS: Central nervous system

CVS: Cardiovascular system

CT: Computerized tomography

DNBA: 7,12-Dimethylbenz(a)anthracene

EGFR: Epidermal growth factor receptor (Her-2 and Her-4)

ER: Estrogen receptor

ERE: Estrogen response element

ERK: Extracellular signal–regulated kinases

ESCC: Oesophageal squamous cell carcinoma

ESR: Erythrocyte sedimentation rate

EST: Estrogen sulfotransferase

GC: Gastric cancer

GI: Gastrointestinal

GBM: Glioblastoma multiforme

GPX1, GPX2 and GPX3: glutathione peroxidases

GSCs: Glioblastoma stem-like cells

GSH: Reduced glutathione

GSK3b: Glycogen Synthase Kinase 3b

HCC: Hepatocellular carcinoma

HDACs: Histone deacetylases

HIF-1 Hypoxia-inducible factor 1

HSP27: Heat shock protein 27

IAPs: Inhibitor of apoptosis proteins

IGF-1: Insulin-like growth factor

IGFBP-3: Insulin-like growth factor-binding protein 3

IκBα: Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha

IL: Interleukin

iNOS: Inducible isoform of nitric oxide synthases

IP3: Inositol triphosphate

ipRGCs: Intrinsically photosensitive retinal ganglion cells

JNK: c-Jun N-terminal kinases

LC: Lung cancer

L-KYN: Lkynurenine

MAPK: Mitogen-activated protein kinase

Mel: Melatonin

MLC: Myosin light chain

MLCK: Myosin light chain kinase

MMP9: Matrix metallopeptidase 9

MT-1 (Mel1a): Melatonin receptor 1

MT-2 (Mel1b): Melatonin receptor 2

MT-3: Melatonin receptor 3

mTOR: Mammalian Target of Rapamycin

MyD88: Myeloid differentiation primary response gene 88

NED: Neuroendocrine differentiation

NF-Kb: Nuclear factor kappa-light-chain-enhancer of activated B cells

NICD1: NOTCH intracellular domain 1

NSCLC: Non-small cell lung cancer

OC: Ovarian cancer

OPN: Osteopontin

PARP: Poly (ADP-ribose) Polymerases

PC: Pancreatic cancer

PCa: Prostate cancer

PDGFRb: Platelet-derived growth factor receptor-beta receptor

PGR: Progesterone receptor

PI3K: Phosphatidylinositol 3-kinase

PKB/ AKT: Protein kinase B

PKC: Protein Kinase C

PRC2/EZH2: polycomb repressive complex 2/ Enhancer Of zeste homolog 2

PUMA: p53 upregulated modulator of apoptosis

RAS: reticular activating system

RNS: Reactive nitrogen species

ROR-α: Retinoid orphan receptor

ROS: Reactive oxygen species

RR: Relative risk

RZR: Retinoid Z receptor

SEEM: Selective Estrogen Enzyme Modulator

SENP1: Sentrin-specific protease 1

SERM: Selective Estrogen Receptor Modulator

SES: Socioeconomic status

SOD: Superoxide dismutases

STAT: Signal Transducer and Activator of Transcription

STS: Steroid sulfatase

SUMO: Small ubiquitin-like modifier

TGFα: Eransforming growth factor

TIMP-1: MMP-9-specific inhibitor

metalloproteinases

TKIs: Tyrosine kinase inhibitors TLRs: Toll-like receptos

TMX: Tamoxifen

TRIF: TIR-domain-containing adapter-inducing interferon-β

VEGF: Vascular endothelial growth factor

VEGFR: Vascular endothelial growth factor receptor

APPENDIX 2: MELATONIN IN CANCER

MELATONIN	Breast	Ovary	Prostate	Gastric oesophageal	Colon	Pancreas	Hepatic	Lung	Leukemia	Glioblastoma
Antiproliferative	yes	yes	yes	ND	yes	yes	yes	yes	yes	yes
Apoptosis	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Cell cycle	Delay (G_0-G_1)	Regulator	ND	ND	Delay	ND	ND	ND	Arrest	ND
Invasion	Decreased	ND	ND	ND	ND	ND	Reduced	Reduced	ND	Decreased
Antioxidant	Yes	ND	ND	ND	Yes	Yes	Yes	ND	Yes	ND
Antiangiogenic	Yes	ND	ND	Yes	Yes	Yes	Yes	ND	ND	ND
Targets	Upregulates: p53, p21	Upregulates: Caspase3	Upregulates: p21	Upregulates: p-HSP27, p-p38, p-JNK, p-ERK	Upregulates: Bax, p16, p21	Upregulates: Caspase3, caspase9, hsp27, hsp70, hsp90	Upregulates: Caspase3, caspase9, p53, cytochrome C, TIMP1	Upregulates: Caspase3, caspase7, Bax, PUMA	Upregulates: Cyclin D1, caspase9, Bax	Upregulates: Caspase3
	Downregulates: ER, VEGF, IL6, TNFa, COX-2	Downregulates: ERK, p38, p-AKT, Bcl2, NF-kB, mTOR, surviving	Downregulates: AR, NF-kB	Downregulates: HIF-1a, VEGF, RZR/ROR, p-AKT	Downregulates: Cyclin A, Cyclin E, COX-2	Downregulates: VEGF, IKBa, NF-kB	Downregulates: MMP9, COX-2, p-AKT, Bcl2	Downregulates: COX-2, HDAC1, Bcl2, p-MLC, Bad	Downregulates: Bmal1, Per1, Per2, Wee, Bcl2	Downregulates: HIF-1a, MMP-2, EZH2, NOTCH1

APPENDIX 3: Melatonin plus chemotherapy in cancer

Breast	Ovary	Prostate	Gastric, oesophageal	Colon	Pancreas	Hepatic	Lung	Leukemia	Glioblastoma
Tamoxifen: Tumor regression Light at night/resistance **Epirubicin:** Tumor regression Enhanced platelet production	**Cisplatin:** Tumor regression Downregulates: hTERT, PTEN/AKT		**Tunicamycin:** Upregulates: Calpain Downregulates: COX-2, E-Cadherin, Snail, Slug **Fluorouracil:** Counteracts: Phosphorylation of MEK, ERK, AKT Enhances cytotoxicity	**Methotrexate:** Ameliorates enteritis **Irinotecan:** Reduces diarrhea **Ursolic acid:** Upregulates: Caspase 3, PARP, cytochrome C Downregulates: MMP9, COX-2, NF-kB **Fluorouracil:** Reduces: p-AKT, PI3K, NF-kB	**Celecoxib:** Better survival Less tumor nodules **Capecitabine/ Fluorouracil:** Slows tumor growth Enhances antioxidant **Cisplatin, Doxorubicin:** Enhances cytotoxicity **Gemicitabine:** Enhances cytotoxicity	**Doxorubicin:** Enhances cytotoxicity, apoptosis Upregulates: caspase3, Bax Downregulates: PI3K, p-AKT Reverses resistance **Cyclo-phosphamide:** Enhances apoptosis Protects from hepatotoxicity **Cisplatin:** Activates PARP, caspase3, caspase9 Downregulates: Bcl2, COX-2, NF-kB **Sorafenib:** Activates PARP, Bax, caspase3, JNK, PINK1 Reduces mitofusin-2 Increases cytotoxicity	**Gefitinib:** Overcomes resistance Decreased p-AKt, p-EGFR, Bcl2, surviving Upregulates caspase3	**Aracytin:** Increases red blood cell count **Puromycin:** Enhances cytotoxicity, apoptosis Upregulates caspase3, PARP, AMPKα Supresses Bcl2, Bcl-xL **Clofarabine, Everolimus, Barasertib::** Enhances apoptosis	**Temozolomide:** Enhances antioxidant effects Reduces proliferation of BTSCs Enhances cytotoxicity Inhibits ABCG2