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Investigation of the effect of melatonin used as a co-treatment with doxorubicin in MCF-7 human breast cancer cells

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Abstract

Melatonin, produced by the pineal gland, has anti-proliferative and cytotoxic effects in many kinds of tumors. Most of its oncostatic actions have been characterized in hormone dependent breast cancer. Melatonin reduces the incidence and growth of mammary tumors in rodents and inhibits proliferation and invasiveness of estrogen responsive breast cancer cells.

Many studies suggest that melatonin is a good co-treatment with conventional chemotherapeutic drugs. It has protective effects in normal tissues palliating side effects and also enhancing cytotoxicity and apoptotic responses induced by chemotherapeutic agents. The molecular pathways modified by melatonin and triggered by chemotherapeutic drugs remain largely unknown.

Doxorubicin is an anthracycline that inhibits DNA and RNA synthesis, currently used in chemotherapy. Melatonin enhances doxorubicin cytotoxicity in different kinds of cancer. In estrogen responsive human cancer cells, melatonin and doxorubicin have synergic effects on apoptosis.

In this study, we demonstrated that physiological concentrations (1nM) of melatonin enhanced the anti-proliferative effect of low doses of doxorubicin in breast cancer cells. Additionally, we identified changes in gene expression induced by doxorubicin at concentrations equivalent to those employed in therapy (1 μM), and found that melatonin reverted the effect of doxorubicin in CDKN1A and TWIST1 expression, two genes altered in breast carcinogenesis.

Key words: Melatonin, breast cancer, doxorubicin, gene expression, MCF-7 cells.

1. Introduction

1.1 Breast cancer

1.1.1. Etiology

Breast cancer is defined as a group of diseases characterized by the accelerated and uncontrolled proliferation and spread of mammary glandular epithelial cells due to mutations, deletions, inversions or translocations in the DNA. These transformed cells are morphologically and functionally immature and aberrant, capable of invading adjacent healthy tissues and disseminating at a distance through the lymphatic system and blood vessels generating metastasis in tissues away from the primary focus.

The tumor genesis is caused by alterations in the DNA that can be either somatic or germinal. Both can take place either in the ductal cells (ductal carcinoma) and lobule cells (lobular carcinoma).

Breast cancer can be due to a combination of circumstances. The main factors increasing the risk include being a woman and getting older. Most breast tumors will develop in women who are 50 years or older. Many women have some risk factors but they will not get breast cancer. By contrary, some women will develop breast cancer without any other risk factors apart from being a woman.

The list of risk factors can be quite extensive, and include modifiable and non-modifiable risk factors (table 1) which influence the origin and development of breast cancer.

NON MODIFICABLE RISK FACTORS	MODIFICABLE RISK FACTORS
Age and gender	**Weight**
Personal and family cancer history	**Physical activity**
Inherited mutations in BRCA1, BCRA2	**Alcohol use**
Early menstruation and late menopause	**Smoking**
High breast tissue density	**Postmenopausal hormone use**
High natural levels of sex hormones	**Radiation exposure**

Table 1.1 *Breast cancer risk factors*

Age: Breast cancer incidence rapidly increases during the reproductive years and after age of 50 (average menopause age) it increases at a slower rate. Most breast cancer are diagnosed in the fifth decade an after.

Sex: Women are more likely than men to develop breast cancer, in a ratio of 99:1 compared to them.

Reproductive years: A women has lower cancer risk the older she began with menstruation. For each year delay in menarche, the risk decreases by around 5%.

Women who experience menopause at a late age are at higher risk of breast cancer. The duration and/or levels of exposure to ovarian hormones have been associated with breast cancer risk. The longer women are exposed to sexual hormones, the higher risk they have to develop breast cancer.

Hormonal therapy for the menopause (HRT): Current or recent past users of hormonal replacement therapy have a higher risk of being diagnosed with breast cancer. The risk increases with estrogen-progestin use more than with estrogen alone. Additionally, there is an association between postmenopausal hormonal treatment and duration of use. The risk diminishes after cessation of the therapy.

Habits: An inverse association of breast cancer incidence with low fat diet has been suggested in studies performed by the Women's Health Initiation randomized control dietary modification trial. Breast cancer rates are higher in countries with high-fat diets than in those countries with a lower fat intake. The risk of breast cancer increases about 10% per 10 grams of alcohol consumed per day, and the tobacco is considered too a risk factor for the development of breast cancer. A moderate physical activity is associated with a lower risk of breast cancer

Radiation: Breast is the most sensitive tissue to the ionizing radiation effects. The increased risk of breast cancer has been well established for women exposed before age 40 years with risk rising from 1.1 to 2.7 at 1 Gy.

Family history and genetic factors: There is a higher risk in patients with first-degree relatives (mother, sister, daughters) previously diagnosed with breast cancer. Familial aggregation can be attributed both to shared environments, similar lifestyles or shared genetic backgrounds. Remarkably, most women with breast cancer do not have a family history of disease. It has been estimated that heritage factors contribute around a quarter, and around three quarters correspond to environmental and lifestyle factors.

High-risk mutations: The mutations in the genes *BCRA1*, *BCRA2, P53, PTEN* and *ATM* have been identified as factors that predispose to breast cancer. Most of the families with four or more breast cancer cases probably bear high-risk alleles, representing around 20-25% of the familial breast cancer risk overall, and around 5% of all breast cancers.

Artificial light at night: Several studies over the last decade have suggested that the recent habit (speaking in terms of generations) of being exposed to artificial light at night (LAN) increases cancer risk, especially for those which growth requires hormones. Women who work night shifts have shown higher rates of breast cancer, whereas blind women, who are not likely to be exposed to or perceive LAN, have shown decreased risks.

Working at night: Women with irregular working hours or who work on rotating night shifts with at least three nights per month, in addition to days and evenings in that month, appear to have a moderately increased risk of breast cancer after extended periods (15 years and over) of working rotating shifts. This fact might be related to the

melatonin pattern of synthesis and secretion, which loses its circadian rhythmicity favouring the appearance of breast tumors [1].

1.1.2. Epidemiology

According to the World Health Organization, breast cancer is the most common cancer among women worldwide; in 2012 nearly 1.7 million new cases were diagnosed. Nowadays, about 1 in 8 women will develop breast cancer along her life in the United States, 1 in 12 in the European Community. The increase on the incidence compared to the rates of 60 years ago (1 in 22), might be explained in terms of the lifestyle changes and the increase on the risk factors. Breast cancer is the first or second leading cause of death depending on the development of the country considered.

In 2017, according to the American Cancer Society, 252,710 women and 2,470 men will be diagnosed of breast cancer in the US. Additionally, 63,410 new cases of *in situ* lesions of the breast will be diagnosed in women.

The incidence rates of breast cancer are higher in developed countries, whereas less developed countries still have low but increasing rates (Figure 2). This regional variation in breast cancer incidence is influenced by a variety of factors, particularly those related to lifestyle. In more developed countries women tend to have less number of children and at older age, the population levels of obesity are higher, the alcohol consumption and hormone replacement therapy use is also more frequent than in less developed countries [2].

The female breast cancer death rate is constantly declining since 1989, and it is due at least in part to development of national trials for early detection enrolling women aged 40-65, the increased awareness and the continually improving treatment options. Also, there has been a gradual reduction in female breast incidence rates among women aged 50 and older coinciding with the decline in prescriptive hormone replacement therapy [3].

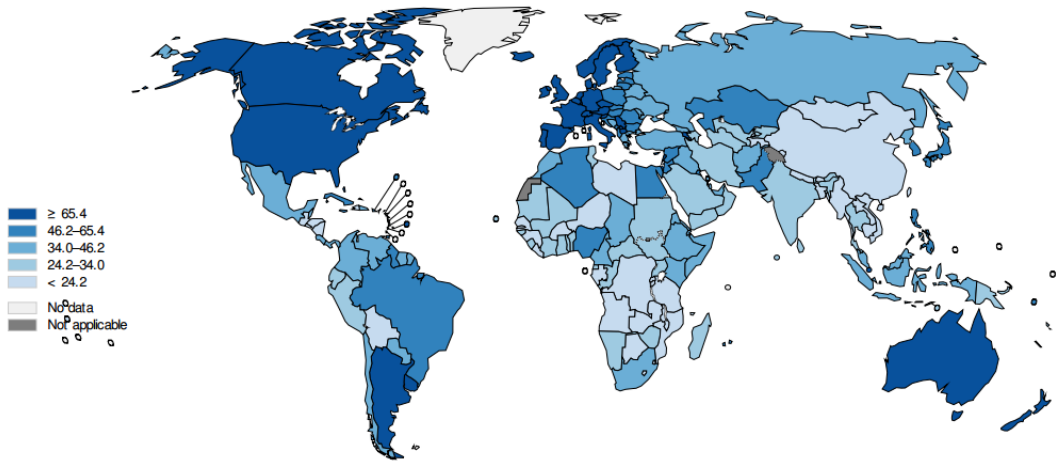

Figure 1. 1 *Estimated age-standardized rates of incident cases, breast cancer, worldwide in 2012.(Available on globocan report, http://globocan.iarc.fr)*

In Spain the annual incidence of breast cancer for females is over 25,000 new cases. In 2014, according to the National Institute of Epidemiology of Spain (INE), the first leading cause of cancer death in females at Spain was breast cancer, with 6,213 deaths, followed by colorectal and lung cancer.

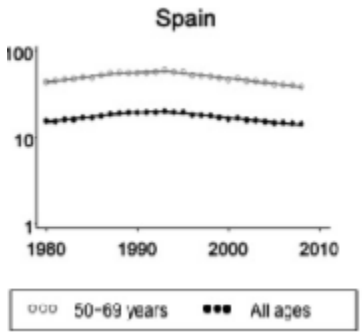

Figure 1.2 *Trends in female breast cancer mortality rates (all ages 50-69)1980-2009. Taken from the descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality. Cancer Epidemiol.*

1.1.3. Estrogens and hormonotherapy in breast cancer

Breast development at puberty and during sexual maturity is mainly stimulated by 17b-estradiol which is the most abundant circulating ovarian steroid and the most biologically active hormone in breast tissue. There is considerable evidence that estrogens are also mammary carcinogens. The role of estrogens in the genesis, growth and progression of mammary cancer is well known for more than one century. Since 1896, when Sir George Beatson demonstrated that ovariectomy caused regression of breast tumors in some premenopausal women patients, one of the principal goals of endocrine breast cancer therapy in the treatment of breast cancer is to neutralize the effects of estrogens in the breast [4].

About 95% breast cancer in women, either in pre- or post-menopausal period, is initially hormone dependent, and the estradiol plays a key role in their development and progression inducing neoplastic transformations similarly to other well-known carcinogens in human mammary epithelial cells. As pointed above, the importance of this ovarian hormone in the genesis of breast cancer is highlighted by the facts that early menarche and late menopause (more years of exposure to estradiol) are associated with a higher risk of breast cancer.

Estradiol binds to the estrogen receptor alpha (ERα) protein in the epithelial cell of the breast. The complex E_2-ERα binds to promoters containing either estrogen response elements (EREs) or AP1 elements. Importantly, in most of mammary tumors at their initial stages, ERα is overexpressed. The hormone and the estrogen receptor complex induce the neoplastic transformation mediating the activation of proto-oncogenes and oncogenes, nuclear proteins and other target genes (figure 4) [5-8].

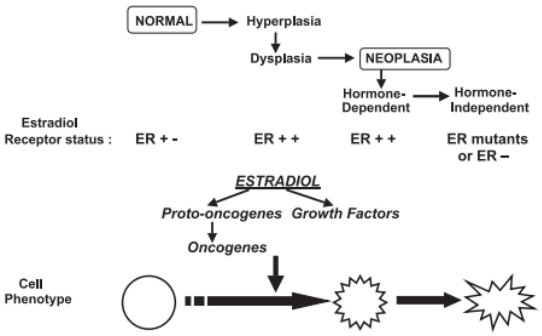

Figure 1.3 *evolutive transformations from normal breast tissue to carcinogen.*
(Taken from Gyneological Endocrionology, October 2007)

Estrogens are mainly synthesized in ovaries during de pre-menopausal period. It is estimated that 70% of estrogens come from the ovaries and 30% from the conversion from androgens, basically dehydroepiandrosterone and dehydroepiandrosterone sulfate, to estrogens in peripheral tissues. In post-menopausal women, though, estrogens are synthesized in peripheral tissues, mainly in adipose tissue, but also in other tissues such as skin or osteoblasts. The fact that adipocites are one of the principal sources of estrogens after the cessation of the follicular function correlates with an increased risk of breast cancer in obese postmenopausal women.

There are three main mechanisms that have been considered to be responsible for the carcinogenicity of estrogens:

1. **The stimulation of cellular proliferation:** Estrogens, through their receptor-mediated hormonal activity, the expression of genes involved in survival, cell proliferation, invasiveness and metastasis are stimulated. This stimulation of the cellular proliferation also favors the increase of the rate in replication errors and point mutations.
2. **Direct genotoxic effects:** By increasing mutation rates through a cytochrome P450 mediated metabolic activation. Redox cycling of estrogens has been shown to generate free radicals which may react to form the organic hydroperoxides needed as cofactors for oxidation to quinones. Those radicals binds covanlently to DNA, which is of particular interest since depurinating adducts are generated from DNA. Therefore, their amount should be correlated to the parallel formation of guanine adducts that are lost from DNA, generating apurinic sites which might play an important role in the cancer initiation process.
3. **Induction of aneuploidy:** In the early stage of mammary tumor lesions aneuploidy patterns are found and estradiol appears to be involved on its induction. Diethylstilbestrol, dienestrol, hexestrol, β-estradiol, ethynylestradiol and estriol caused significant increase in aneuploidy within a narrow range of high concentrations. Estrogens may have a mechanism of mitotic arrest similar to that of colchicines, by inhibiting the polymerization of tubulin to form microtubules. The interaction between estrogens and microtubules may

mediate the induction of aneuploidy in somatic cells. Aneuploidy induction by may be related to their carcinogenic potential [6].

About two thirds of the patients diagnosed with breast cancer have positive hormone receptors (estrogen receptors alpha and alternatively spliced derivatives, progesterone receptors or both), that means that they depend on estrogens to growth. The hormonotherapy is based in pharmacological strategies to selectively neutralize the effects of the estrogens on the breast. The employed modulators of the estrogenic action are:

SERMs (Selective Estrogen Receptor Modulators): They antagonize the estrogen receptor at the nuclear level preventing the estrogen binding to its receptor, thus impairing by this way many physiological actions of the estrogens receptors in breast cancer malignant transformed cells, but can still potentially maintain their beneficial effects in other tissues such as in bone and cardiovascular system. The prototypal drug of this class is Tamoxifen which behaves as an estrogen agonist in breast cancer cells. Tamoxifen binds to the estrogen receptor promoting a conformational change in its structure different from that triggered by estradiol, and as consequence, the tamoxifen-ERα complex recruits repressors instead of coactivators to the target promoters. Tamoxifen behaves as an agonist in other tissues such as endometrium which is a problem because it can cause endometrium cancer. Another therapy used in breast cancer with positive hormone receptor is the Fulvestrant, which blocks the mediated transcription and increases the degradation of the estrogen receptors without agonist effects in other tissues, conferring benefits over other SERMs like tamoxifen. After many trials, use of fulvestrant in postmenopausal women has eventually being approved.

SEEMs (Selective Estrogen Enzyme Modulators): The SEEMs are inhibitors of the enzymatic mechanisms involved in the formation and transformation of precursors into active estrogens in breast cancer cells; resulting on the inhibition of enzymes involved in the steroid synthesis or activating the enzymes that inhibit estrogens. Two examples of Selective Estrogen Enzyme Modulators include both steroidal (formestane and exemestane) and non-steroidal (such as anastrozole and letrozole), drugs that act as inhibitors of the enzyme complex aromatase, the main enzyme involved in the synthesis and regulation of local estrogen production [7, 8].

1.1.4. Chemotherapy for breast cancer

Chemotherapy consists in the use of one or more anti-cancer drugs. Chemotherapeutic agents can be administered intravenously or orally but these compounds are usually applied by intravenous infusion because through the bloodstream they reach growing cancer cells characterized for their high rate of self-renewal activity, which is a particular property of malignant cells.

As for many other kinds of cancer, chemotherapy can be given to breast cancer patients. Chemotherapeutic drugs are administered both to treat early-stage invasive breast cancer, with the purpose to get rid of any cancer cells that may be left behind after surgery, trying to reduce as much as possible the risk of cancer recurrence, and

advanced-stage breast cancer, to destroy and eliminate as many malignant cells as possible. According to the moment of administration, we speak of adjuvant chemotherapy, used after surgery, and neoadjuvant chemotherapy, administered before surgery. With surgery the tumor is dissected and what adjuvant therapy cancer does is to try to eliminate those cells not removed, to avoid their proliferation and their ability to spread out [9, 10].

In case of use neoadjuvant therapy, applied prior to surgery, the main benefits pursued are the decrease in the size of the tumor, requiring less extensive surgery and the tumor removal easier. Moreover, administering chemotherapeutic drugs prior to the tumor removal will help the monitoring of the disease in case of drugs which do not diminished the size of the tumor, allowing to considerate other cocktail of compounds to be considered. The treatment of mammary tumors detected on early states commonly use a combination of drugs, whereas in advanced breast cancer is more common the treatment with a unique chemotherapy molecule [10].

The way to administrate chemotherapy is based on cycles, which combines periods of administration with resting periods, allowing the body to recover and therefore minimizing the effects derived from treatment. As said above, patients may be given one or more anti-cancer molecules that kill cells in different ways and at different phases along the cell cycle.

Attending to their chemical structure and the molecular pathways targeted for these chemicals, we can distinguish several kinds of chemotherapeutic agents [11, 12]:

1. **Alkylating agents:** These drugs are the oldest and at the same time the most commonly used molecules. They work by directly damaging DNA by attaching an alkyl group to guanines. They are used to treat a wide variety of cancers, but have the greatest effect on those that are slow-growing. They kill malignant cells in any phase of the cell cycle. As examples, we can mention cisplatin and carboplatin.
2. **Antimetabolites:** They are chemotherapy agents that inhibit processes required for DNA or RNA synthesis. They are cell-cycle specific. For example, gemcitabine is phosphorylated and transformed into dFdCTP, which is incorporated on the end of the elongating DNA strand. This allows the addition of just one more deoxynucleotide and thereafter, the DNA polymerases are unable to proceed.
3. **Plant Alkaloids**: Obtained from certain types of plants found in nature, they belong to the family of compounds also known as microtubule inhibitors (MIs). MI agents include microtubule depolymerizing compounds (*Vinca* alkaloids) and polymerizing agents (taxanes). In both cases their cytotoxicity is due to their capability to interfere with tubulin, impairing the formation of the mitotic spindle apparatus and therefore, leading to mitosis disruption. Most plant alkaloids are cell-cycle specific, but can cause damage in all phases. Among *Vinca* alkaloids we can mention vincristine, and vinorelbine. The most commonly used taxanes are paclitaxel and docetaxel
4. **Anthracyclines:** Anthracyclines are anti-tumor antibiotics whose mechanism of action is based on their ability of insertion between two DNA strands, resulting

in a DNA-anthracycline complex that inhibits both DNA and RNA synthesis. Additionally, this mechanism also targets DNA for cleavage by topoisomerase II, leading to cellular cascades that eventually results in cell death. In breast cancer, they are commonly used in chemotherapy combined with other drugs in patients who have had surgery to remove the tumor. Epirubicin and doxorubicin are probably the most commonly used anthracyclines. As doxorubicin is the drug used in this work in combination with melatonin, we will describe below this compound a bit more in detail.

5. **Molecular targets:** Other current approaches more recently developed include compounds recently emerging that target specific molecules or pathways. Many of them are monoclonal antibodies that increase the immune response to tumor cells. For example, trastuzumab is a monoclonal antibody indicated in breast cancer that targets the oncogene human epidermal growth factor (HER2/neu). Lapatinib , is a reversible inhibitor of the tyrosine kinase activity of the epidermal growth factor receptor (EGFR).

1.2 Doxorubicin

1.2.1. Synthesis

Doxorubicin (doxorubicin hydrochloride) is a cytotoxic anthracycline antibiotic used as an effective agent against a wide range of malignant conditions. This antibiotic was first time isolated from the *Streptomyces peucetius var. caesius* in the 1970's [13].

The structural formula is shown in figure 5. Doxorubicin consists of a naphathacene quinona nucleus linked through a glycosidic bond at ring atom 7 to an amino sugar, daunosamine. This molecule is amphoteric, with an acidic function in the ring phenolic group. The lipophilic ring from the antracycline is highly hydrophobic, but it is surrounded by five hydroxyl groups producing a hydrophilic center. This property allows doxorubicin to bind to cell membranes as well as plasma proteins.

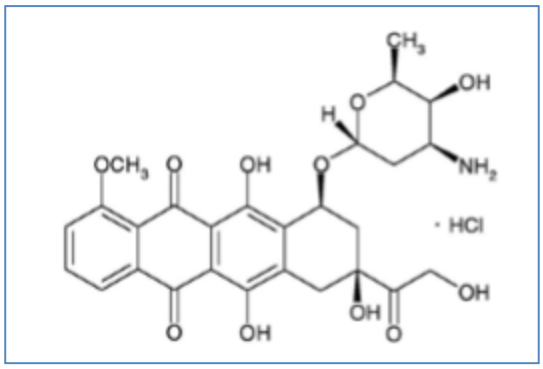

Figure 1.4 *Doxorubicin structural formula*

1.2.2. Mechanism of action

Doxorubicin enters into the cells by diffusion. Anthracyclines are known to intercalate into DNA in vitro, and crystal structures of DNA and Doxorubicin have been obtained. As consequence, doxorubicin causes DNA breaks and results in inhibition of both DNA and RNA polymerase, ceasing DNA replication and DNA transcription, therefore interfering with DNA synthesis. DNA-Doxorubicin interaction is related to the poisoning of topoisomerase II (TOP2A) but not topoisomerase I. Translocation of doxorubicin into the nucleous is thought to occur via its binding to the ubiquitin proteasome system. Subsequent topoisomerase II cytotoxicity is likely originated through the mismatch repair genes MSH2 and MLH1 [14, 15].

Other doxorubicin actions are the generation of free radicals causing more DNA damage. This anthracycline undergoes a one-electron reduction to form a semiquinone radical. Re-oxidation of this compound back to doxorubicin leads to the formation of reactive oxygen species (ROS). Another effects of doxorubicin are the inhibition of the macromolecule production, the DNA unwinding and the DNA alkylation levels. Recent studies have also demonstrated that doxorubicin is capable to intercalate into mitochondrial DNA too. It can affect the cell membrane by causing an enzymatic electron reduction by binding to plasma proteins causing the formation of free radicals. All of these mechanisms make doxorubicin a potent anticancer drug, but they are also responsible for the dangerous side effects [16].

1.2.3. Adverse reactions

Doxorubicin side effects depend on the dosage and the capacity of regeneration of the patient's bone marrow. This antrhracycline drug is not targeted specifically to the tumor, affecting to the growth of many other cells and results in the inmunosupression of the immune system. This side effect makes the patient more susceptible to fatigue, microbial infections and longer healing times.

In addition to side effects common to other types of chemotherapeutic agents that include acute vomiting, nausea, gastrointestinal problems and disturbances of the neurological system between others, doxorubicin acts inducing toxicity in most of the major organs including heart, brain, liver and kidneys.

Cardiotoxicity is the greatest risk of doxorubicin-induced toxicity. This cardiotoxicity is the result of the enlargement of the cardiomiocytes and the consequent hypertrophy, caused by the brain natriuretic peptide (BNP) and the atrial natriuretic peptide (ANP) that are highly expressed in response to this anthracycline. Another way for cardiotoxicity is due to the doxorubicin interaction with the mitochondrial DNA in cardiac muscle [17].

Doxorubicin is incapable of crossing the blood-brain barrier. In the central nervous system, its toxicity is caused by indirect mechanisms that the production of TNF-α in excess resulting in cell death via apoptosis. Additionally, over 40% of the patients suffer some form of liver injury. The problem with the toxicity induced by doxorubicin includes the decrease of inorganic phosphate (including ADP, ATP and AMP) causing pathological conditions in hepatocytes and decreasing the cell's ability to perform

energy-dependent tasks. In the kidneys doxorubicin acts causing nephropathy, hyperlipemia and proteinuria by injuring glomerular podocytes due to the interaction between the drugs and the normal functioning of the mitochondria [18, 19].

Finally, concerning cancer cells, the last but not least important problem of doxorubicin that limits its use is resistance. The mechanism of resistance involves the protein transporters ABCB1 and ABCC1. These molecules are ATP-dependent drug efflux pumps that causes increased drug efflux from the cells, resulting in lower levels of exposure to cytotoxic drugs [20].

1.3 Melatonin

1.3.1. Synthesis and secretion

Melatonin (N-acetyl-5-methoxytrypamine) is a low molecular weight indolic hormone that was initially isolated from and chemically identified in bovine pineal tissues by the dermatologist Aaron Lerner in 1958.

Pinealocytes, the main cells contained in the pineal gland, synthesized melatonin from tryptophan taken up from circulation and transformed to serotonin. Melatonin is converted from serotonin by a process that involves the sequential activities of two enzymes, serotonin-N- acetyl transferase (NAT) and hydroxyindole-O-methyl transferase (HIOMT). It is mainly synthesized from the pineal gland but it is synthesized too in extra-pineal organs including the retina, ovary, placenta, cerebellum, gut mucosa, airway epithelium, adrenals or thyroid.

Melatonin production and secretion are regulated by photoperiod; the pineal hormone is synthesized in response to the onset of darkness. Both production and secretion are repressed by light during the daytime and induced in response to darkness at night. The melatonin secretion rhythm is apparent in humans at 6 months of age, and the magnitude of it nocturnal secretion peaks in early childhood. Both the secretion of melatonin and the size of the pineal gland decrease with age.

Melatonin synthesis also involves the nervous system. The photoperiodic information that modulates the circadian synthesis and secretion of melatonin is processed in the endogenous biological clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus. Nerve fibers in the retinohypothalamic tract connect the retina to the suprachiasmatic nucleus (SCN). The SCN stimulates the release of Norepinephrine from sympathetic nerve fibers from the superior cervical ganglia that synapse with the pinealocytes. Norepinephrine causes the production of melatonin in the pinealocytes by stimulating the production of cAMP. Because the release of norepinephrine from the nerve fibers occurs at night, this system of regulation maintains the body's circadian rhythms [21].

1.3.2. General actions

The best described melatonin action is its chronobiotic action regulating the light and dark cycle. This information depends on circulating levels of melatonin. The pineal gland sends information to the rest of the body; melatonin is highly lipophilic and

rapidly diffuses into the blood stream. This information depends on circulating levels of melatonin. In the dark, synthesis and circulating levels of melatonin are highs, and when it is light these levels are diminished. These changes on melatonin circulating levels are received by cells expressing melatonin receptors and sense by this means whether it is light or dark.

Melatonin has anti-oxidative actions too since it is a free radical scavenger. Melatonin's functional repertoire in terms of limiting molecular destruction by both oxygen and nitrogen-based radicals and associated metabolites is highly diverse. The pineal hormone exerts several actions at different levels to aid in the ability of organisms to resist the damage normally inflicted by radicals and radical-related products. Thus, melatonin reduces free radical generation at the mitochondrial level in a process generally referred to as radical avoidance. The pineal hormone stimulates several antioxidative enzymes that convert highly toxic species to innocuous products. Additionally, melatonin promotes the synthesis of another antioxidant, glutathione, and inhibits at least one enzyme, nitric oxide synthase, that normally produces free radicals, in this case nitric oxide and its derivatives (NO). Melatonin acts against molecular damage in all regions of the cell, protects against lipid peroxidation, protein mutation and against mitochondrial and nuclear DNA damage by radicals and they related reactants. Not only does melatonin carry out these functions to help cells avoid molecular damage, but it also directly neutralizes free radicals. In addition to melatonin itself, several of the metabolites that are formed when melatonin scavenges radicals also participate in antioxidative defense by incapacitating toxic species and by modulating the activities of enzymes that maintain the redox balance within cells. Some of the antioxidative actions of melatonin, e.g., stimulation of antioxidative enzymes are likely receptor-mediated while others are receptor-independent, e.g., direct radical scavenging [21-23].

1.3.3. Melatonin and estrogens

Based on the role of melatonin in inhibiting gonadal maduration and sex hormone production and secretion in mammals, it has been proposed a possible relationship between pineal function and the etiology of breast cancer. Tamarkin and colleagues found that the amplitude of the nighttime peak of melatonin is diminished in women with estrogen positive breast cancer in comparison with either estrogen negative cancer or healthy women [24].

Since then, many studies have addressed the positive role of melatonin in breast cancer. The pineal hormone is thought to work primarily through two mechanisms; first, the down regulation of the hypothalamic-pituitary axis, and second, through direct actions at the tumor cell level. Acting at the hypothalamic-pituitary axis melatonin can act as a regulator of the circulating estrogen levels. There is an inverse relationship between melatonin and estrogen levels; thus, women exposed to light at night, which suppress the melatonin production, show increased levels of estrogens in plasma. Experiments *in vitro* have demonstrated that treatment of human granulose cells with melatonin results in a reduction of estradiol secretion [25].

Melatonin is a molecule that behaves both as a SEEM and as a SERM. In postmenopausal women, estrogens are synthesized in the mammary tissue by transformation from androgens of adrenal origin. Many studies, most of them carried out in vitro on the MCF-7 human breast cancer cell line, have demonstrated the direct effects of melatonin as a SEEM on the control of the enzymes involved in the biosynthesis and transformation of estrogens. One of the major pathways activated in the synthesis of estrogens in mammary tumor cells is the aromatization of androstenedione to estrone by the enzyme complex aromatase. Melatonin is able to inhibit both the aromatase activity and expression on basal conditions and when aromatase in stimulated by cAMP. This modulator action of melatonin has been also described in rats bearing DMBA-induced mammary tumors. Melatonin also reduces the activity and expression of estrogen sulfatase and 17beta-hydroxysteroid dehydrogenase and increases the activity and expression of estrogen sulfotransferase, therefore protecting mammary tissue from excessive estrogenic effects. Importantly, melatonin modulates the expression and activity of all these enzymes not only in tumor cells, but also in surrounding cells located in the proximity of the malignant epithelial cells, such as fibroblasts and endothelial cells [25, 26].

The indolic hormone interferes with the effects of the endogenous estrogens behaving as a SERM. Unlike other antiestrogenic molecules such as tamoxifen, melatonin does not directly bind to ER. In MCF-7 estrogen responsive breast cancer cells, melatonin diminishes the expression of ERα and inhibits the estrogen-dependent transcriptional activation through destabilization of the E2-ER complex from binding to DNA in both estrogen response element (ERE) and AP1 containing promoters. This effect is likely mediated by calmodulin, since melatonin acts as a calmodulin antagonist inducing conformational changes in the ERα-calmodulin complex thus facilitating its binding to an estrogen response element (ERE). The effects of melatonin might also been be triggered by its binding to its specific membrane receptors (MT1) resulting in a crosstalk with the ER- signalling pathway. A link connectiong both signalling pathways (melatonin and estradiol) could be the opposite effects that they cause over cAMP intracellular concentrations [27-29].

1.3.4. Melatonin and cancer

As pointed above, melatonin has indirect oncostatic effects that include modulation of endocrine and immune system, anti-proliferative, pro-apoptotic actions and antiangiogenic actions (figure 7). Melatonin acts downregulating the hypothalamic-pituitary and is involved in the oncostatic action in the case of sex hormone-dependent tumors like the breast cancer. Another known action is the modulation of the immune system. Melatonin modulates positively the cytokine production and the lymphocyte proliferation, exerting the immuno-enhacing activity [30].

It has recently been shown that the vascular endothelial growth factor (VEGF), considered the most important humoral activator in tumor process, is decreased in the blood levels on patients treated with melatonin. The antioxidant potential of melatonin diminishes indirect and direct damage of DNA due to the excessive free radical generation in the mechanism of carcinogenesis [31].

Various clinical trials have been performed to evaluate the potential beneficial effects of melatonin in human neoplasms. The main conclusion is that melatonin, concomitantly added in conjunction with chemotherapy or radiotherapy, may serve a beneficial rile in cancer patients who are treated with chemotherapeutic drugs, since patients who received melatonin experienced substantial improvements, particularly in terms of tumor remission, 1-year survival rates and ameliorating the side effects[32].

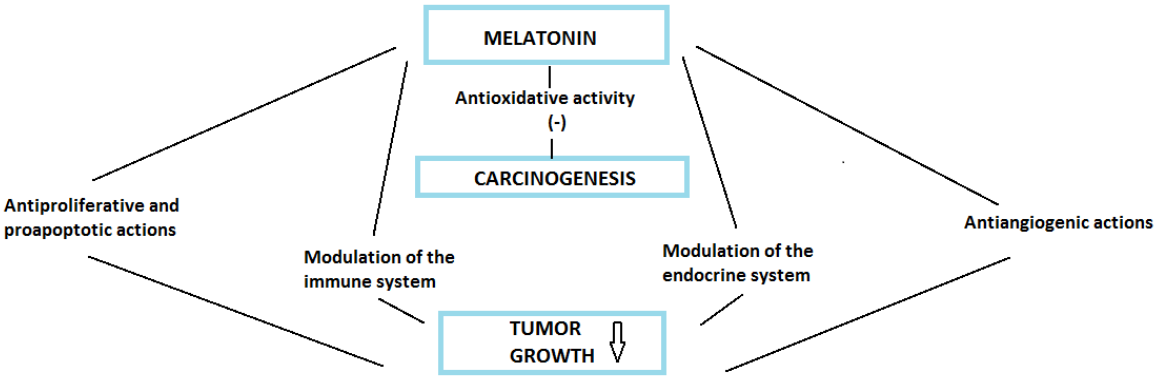

Figure 1.5 *Melatonin oncostatic actions scheme. (Reproduction from J.R. Pasqualini Biochimica et Biophysica Acta 1654)*

1.3 Melatonin and doxorubicin in cancer

In some of the clinical trials mentioned above, melatonin was administered to patients receiving doxorubicin. Melatonin was capable of increasing the efficacy of chemotherapy potentiating the induced cytotoxicity and the apoptosis induced by the anthracycline. Both melatonin and doxorubicin work synergistically inhibiting the cell growth and inducing the apoptosis. In human hepatoma cell lines the treatment with melatonin had an inhibitory effect on cell proliferation. When combined with doxorubicin, the growth inhibition was potentiated and cell apoptosis was induced. Other studies provided evidence that *in vitro* melatonin enhances the pro-apoptotic and cytotoxic actions of doxorubicin in rat pancreatic tumors [33, 34].

These effects had also been proved in patients. According to the study of Lissoni et al in 250 metastatic solid tumors patients, the administration of the melatonin concomitant with doxorubicin reduces the toxicity and enhances the chemotherapy effects, increasing the survival time on patients and the tumor regression. Melatonin had also a protective action against chemotherapy toxicity in these patients, decreasing the side effects thrombocytopenia and neurotoxicity [35].

1.3.1 Melatonin and doxorubicin in breast cancer

In breast cancer melatonin acts as both a tumor metabolic inhibitor and an inhibitor of circadian regulated kinases. A recent report of Xiang and collaborators suggests that one of the reasons behind doxorubicin resistance in breast cancer is the disruption of the circadian melatonin signal by light at night. The pineal hormone reestablishes the sensitivity of breast tumor to doxorubicin triggering tumor regression.

Melatonin combined with chemotherapy may increase the therapeutic effects of doxorubicin. According to Koşar et al, melatonin increases the apoptosis of MCF-7 cancer cells by exerting a synergic effect with doxorubicin. The intracellular production of reactive oxygen species, mitochondrial membrane depolarization, apoptosis level, procaspase 9 and PARP activities, and caspase 3 and caspase 9 activities were higher in the doxorubicin plus melatonin groups than in untreated control cells, once again pointing to melatonin as a supplementary drug in the treatment of cancer. However, to date there is limited knowledge about the interplays of melatonin and chemotherapy, particularly doxorubicin on molecular aspects such as gene expression profile and gene post-translational modifications [36, 37].

2. Objectives

Melatonin has oncostatic effects in hormone-dependent mammary tumors like breast cancer by acting at different levels: causing a down-regulation of the hypothalamic-pituitary, modulating the enzymes necessary for the synthesis of estrogens and regulating the signaling pathways triggered by estrogens in the malignant cells. Melatonin enhances the cytotoxic effects of chemotherapy in many cancer models. Nevertheless, little is known about the changes in gene expression induced by chemotherapeutic agents in breast cancer cells in some genes known to be altered in breast cancer development and progression.

The objectives of this work were:

a) This work is intended to be a state of the art review of the current knowledge on breast cancer, melatonin and chemotherapy.

b) Using the estrogen responsive MCF-7 human adenocarcinoma cell line, the objectives were:

- To characterize the changes in gene expression induced by doxorubicin on a series of genes known to be altered in breast cancer.
- To establish whether melatonin induces changes in the expression of the same genes.
- To verify if melatonin is able to modulate the changes in expression induced by doxorubicin
- To test the ability of melatonin to enhance the anti-proliferative effects of doxorubicin used at sub-pharmacological doses.

c) In summary, to demonstrate that melatonin can be a useful as a co-treatment with doxorubicin in breast cancer.

3. Materials and methods

3.1. Cells and culture conditions

The cells used in this work were MCF-7 human breast cancer cells, purchased from the American Tissue Culture Collection (ATTC nº HTC-22TM) (Rockville, MD, USA). They were maintained in 75 cm^2 plastic culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Madrid, Spain) supplemented with 10% fetal bovine serum (FBS)(PAA Laboratories, Pasching, Austria), penicillin (20 units/ml) and streptomycin (20µg/ml) (Sigma-Aldrich, Madrid, Spain). The cells grew as monolayer cultures at 37°C in a humid atmosphere containing 5% CO2.

Before each experiment, cells were transferred to phenol red-free Dulbecco's medium containing 0.5% dextran-charcoal stripped FBS (scFBS), and maintained for 3 days. Then cells were washed twice using DMEM, and they were detached from the culture plate with a 0.25% trypsin containing solution.

After neutralization, cells were transferred to a 15 ml conical tube, centrifuged at 100 x g for 5 minutes and the pellet was resuspended in culture media removing a sample for counting.

3.1.1. Determination of cell number

We used a hemocytometer (Marienfeld, GmbH) to determine the total number of cells and its variability. Briefly, 100 µl of the cell suspension were pipetted into the counting chamber, underneath the coverslip, allowing capillary action to draw it inside.

On a hemocytometer the full grind contains nine squares, each of which is 1 mm^2. The central counting area of the hemocytometer contains 9 large squares and each large square has 16 smaller squares. We used a fluorescence microscope (NIKON TMS) to calculate the number of cells in the initial cell suspension by the formula showed in figure. Alive cells which were situated inside the four corners of the square and also in the central square were counted.

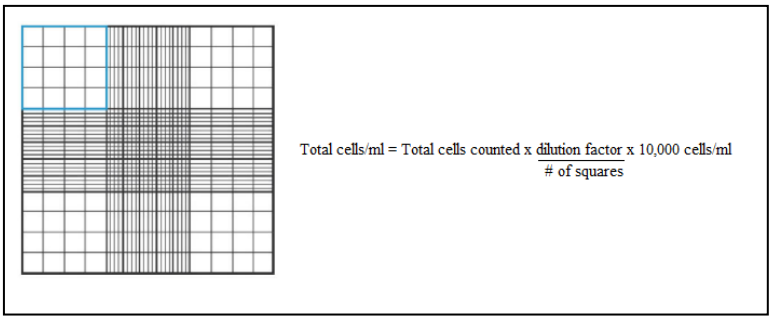

Figure 3. 1 *Hemocytometer gridlines. Hemocytometer diagram with one of the 1 sheets of 16 squares used for counting underlined*

3.2. Reagents and treatments

Doxorubicin (Sigma-Aldrich, Spain) is an anthracycline anti-cancer drug that causes DNA breaks and results in inhibition of both DNA and RNA polymerases, interfering with DNA and RNA synthesis. Doxorubicin was diluted in ethanol at a final concentration of 0.1 M (stock) and stored refrigerated. When this drug was added to the culture media, the final concentration of ethanol was lower than 0,001%.

Melatonin stock (Sigma-Aldrich, Spain) was diluted also in ethanol at a final concentration of 100 mM and stored aliquoted at -20ºC. Melatonin was used at a final concentration of 1 nM in the culture media, which a dose considered equivalent to the physiologic concentration is found in humans during the night period.

3.3. Gene expression studies

For the gene expression studies, MCF-7 cells were seeded in 60x15 cm dishes at a density of $8x10^4$ cells per dish, in DMEM supplemented with 10% FBS. After 24 h incubation, to allow cell attachment, media were replaced by fresh ones containing 1 µM doxorubicin, 1 nM melatonin, doxorubicin plus melatonin or vehicle (ethanol at a final concentration lower than 0.001%). Cells were incubated for 6 hours at 37°C before total cellular RNA was extracted.

3.4. RNA isolation and cDNA synthesis

The total cellular RNA was isolated from MCF-7 cells and purified with the Nucleospin RNA II kit (Macherey-Nagel, GmbH & Co., Germany) following the manufacturer's instructions (figure 3.2). Briefly, cells were lysed by incubation in a solution containing large amounts of chaotropic ions. This lysis buffer immediately inactivated RNases and created appropriate binding conditions which favour adsorption of RNA to the silica membrane. After lysis, homogenization and reduction of viscosity were achieved by filtration with NucleoSpin Filter units provided with the kit. After that, contaminating DNA was removed by an rDNase solution which was directly applied onto the silica membrane during the preparation. Total RNA were finally eluted with RNase-free water, aliquoted and storaged at -80°C. The quality and quantity of the RNA eluted were measured with and spectrophotometer (Nanodrop 1000 V 3.6). The absorbance ratio A260 nm/A80 nm was always greater than 1.9.

For cDNA synthesis, 0.5 µg of total RNA was used as template using the RT2 First Stand Kit (Qiagen, USA), following the manufacturer´s instructions. First, the genomic DNA was eliminated by incubating the samples 5 min at 42°C. After mixing with the reverse transcription mix, the samples were incubated for exactly 15 min at 42°C in a final

volume of 20 µl. The reaction was stopped by incubating at 95°C for 5 min. 91 µl of RNA-free water was added to each reaction and samples were kept on ice until proceeding with the real-time PCR protocol.

COMPONENT	AMOUNT
RNA	**0,5 µg (*n* µl)**
Buffer GE	**2 µl**
Buffer BC3	**4 µl**
Control P2	**1 µl**
Reverse transcriptase Mix	**2 µl**
Nuclease free water	**Up to 111 µl**

Table 3.1 *Components used in the reverse transcription reaction*

3.5. RT2 Profiler PCR Array

Pathway-focused gene expression profiling was performed using a 96-well human breast cancer PCR array (RT2 Profiler PCR array – PAHS-131ZA, Human Breast Cancer PCR Array, Qiagen, USA). In this array, each well contained all the components required and designed to ensure that each quantitative real time PCR reaction (qRT-PCR) will generate single, gene-specific amplicons, preventing the co-amplification of non-specific products.

The genes tested are involved in breast cancer and encode important proteins that contribute to apoptosis, metabolism, DNA repair, the cell cycle, growth factors, hormone receptors, transcription factors, etc. The assay was used to monitor the expression of 84 genes related to breast cancer pathways, plus 5 housekeeping genes. The housekeeping genes are indicated by Qiagen since they do not present alterations on expressions in all the experiments. For human breast cancer PCR array the housekeeping genes indicated were: actin beta (ACTB), β-2-microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine phosphoribosyl-transferase 1 (HPRT1) and ribosomal protein, large, P0 (HPLOP0).

Each RT^2 Profiler PCR array plate also includes control elements for data normalization, genomic DNA contamination detection, RNA sample quality and general PCR performance (figure 10). The PCR array performs gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray.

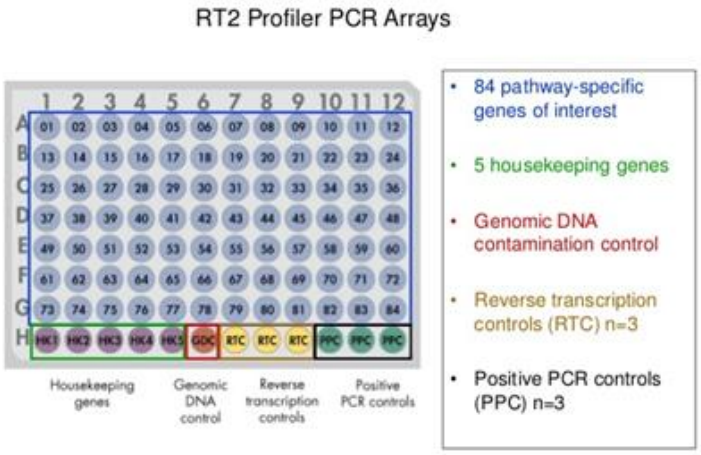

Figure 3.1 *Scheme of a RT2 Profiler™ PCR Array (Qiagen)*

The cDNA template (102 µl) were mixed with the appropriate ready-to-use 2X PCR master mix (1350 µl), aliquoted 25 µl to each well of the same plate, and then the real-time PCR cycling program were performed in an MX3005P (Agilent, CA, USA) using Brilliant® III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, CA, USA) following the manufacturer's instructions. Amplifications were initiated by 1 cycle at 95°C for 10 min and then performed for 45 cycles for quantitative analysis using the following temperature profile: 95°C for 30 sec (denaturation); 60°C for 60 sec (annealing/extension), setting the instrument to detect and report fluorescence at each cycle during the 60°C annealing/extension step. Melting curves were performed by using dissociation curve to verify that only a single product with no primer-dimers was amplified.

3.5.1. qPCR Data analysis

The $\Delta\Delta C_T$ method is recommended for data analysis. In separate reactions, the C_T values for the housekeeping gene(s) (HKG) and for the genes of interest (GOI) were determined in each sample. It is important to note that only CT values less than 35 were used for the analysis.

For each pair-wise set of samples to be compared, calculate the difference in ΔC_T values (ΔΔCT) for the genes of interest between the two samples. For each sample, the difference between the C_T values (ΔC_T) for each gene of interest and the average CT value of the set of housekeeping genes were calculated as follows:

- ΔC_T (control) = C_T (GOI) – C_T (HKG)
- ΔC_T (experimental) = C_T (GOI) – C_T (HKG)

Secondly, the difference in ΔC_T values ($\Delta\Delta C_T$) for the genes of interest between the two samples were calculated as follows:

- $\Delta\Delta C_T$ = ΔC_T (experimental) – ΔC_T (control)

Finally, the fold-change for each gene between control group and the different treatments were calculated as $2(-^{\Delta\Delta CT})$.

3.6. Measurement of *BCLA2*, *CDKNA1*, *GATA3*, *MUC1*, *SERPINE1*, *SNAI2* and *TWIST1* mRNA gene expression

The specific analysis of the *BCLA2, CDKNA1, GATA3, MUC1, SERPINE1, SNAI2* and *TWIST1* mRNA gene expression in MCF-7 cells was carried out by qRT-PCR. Primers used for amplification of these genes were designed so that the coding sequence is interrupted by at least one intron in the gene. The primers were purchased from Sigma (Sigma-Aldrich, Madrid, Spain)

For the primer design the following programs were used: SequenceAnalys*is 1.6,* DAMAN *(Lynnon Corporation); SequenceManipulationSuite*. In order to optimize primer concentration, PCRs with a matrix of six concentrations of each pair (50 nM to 400 nM) were performed. RT-PCRs were performed in an MX3005P (Agilent, CA, USA) using β-actin as a housekeeping gene. Each reaction was run at least in triplicate. Melting curves were performed by using dissociation curve to verify that only a single product with no primer-dimers was amplified. For the primers used there were no differences between transcription efficiencies, and the fold-change in each experiment was calculated by the $2^{-\Delta\Delta Ct}$ method. The oligonucleotides used are shown in table 3.

GENE	PRIMER SECUENCE
β-actin Forward	5´-TCCTGCGAGTGCTGTCAGAG-3´
β-actin Reverse	5´-TCACCGCCCTACACATCAAAC-3´
CDKN1A (p21) Forward	5´-CAGCATGACAGATTTCTACC-3´
CDKN1A (p21) Reverse	5´-CAGGGTATGTACATGAGGAG-3´
TWIST1 Forward	5´-CTAGATGTCATTGTTTCCAGAG-3´
TWIST1 Reverse	5´-CCCTGTTTCTTTGAATTTGG-3´
MUC1 Forward	5´-GCAAGAGCACTCCATTCTCAATT-3´
MUC1 Reverse	5´-TGGCATCAGTCTTGGTGCTATG-3´
SERPINE1 Forward	5´-ATCCACAGCTGTCATAGTC-3´
SERPINE1 Reverse	5´-CACTTGGCCCATGAAAAG-3´
GATA3 Forward	5´-CGGTCCAGCACAGGCAGGGGT-3´
GATA3 Reverse	5´-GAGCCCACAGGCATTGCAGACA-3´
SNAI2 Forward	5´-TCACCGCCCTACACATCAAAC-3´
SNAI2 Reverse	5´-TCACCGCCCTACACATCAAAC-3´
BCL2 Forward	5´-CCTTTGGAATGGAAGCTTAG-3´
BCL2 Reverse	5´-GAGGGAATGTTTTCTCCTTG-3´

Table 3.2 *Primers used for amplifications of mRNA transcripts.*

3.7. Cell proliferation assays

Cells were initially cultured for 24 hours in DMEM supplemented with 0.5% dextran-charcoal stripped FSB (csFBS) before being seeded into 96-multiwell plates in DMEM supplemented with 10% FBS and incubated at 37^{o}C for 24 hours to allow for cell attachment. Melatonin pre-treated cells were incubated for 24 hours in DMEM supplemented with 10% FBS containing 1 nM melatonin before being seeded into 96-multiwell plates. In both cases, the media were replaced by fresh ones with 10% FBS and containing doxorubicin at different concentrations (1 µM, 100 nM, 10 nM, 5 nM, 1 nM, 0, 1 nM), in the absence or presence of 1 nM melatonin (Sigma-Aldrich, Madrid, Spain) and/or vehicle (ethanol at a final concentration lower than 0.0001%). Cells were cultured for 5 days and after that time cell proliferation was measured by the MTT [3(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] method, reading absorbance at 570 nm in a microplate reader (Labsystems Multiskan RC 351, Vienna, VA, USA). MTT was obtained from Molecular Probes Inc (Eugene, OR, USA). Briefly, yellow MTT (5 mg/ml in PBS) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells. The formazan crystals can be dissolved by adding 4 mM HCl. The plate is reader after 24 h in a spectrophotometer. An increase in cell number is directly related to the increase in absorbance due to the amount of MTT formazan formed.

3.8. Statistic analysis

The results of this study are expressed as the mean ± standard error of the mean (SEM). The statistical differences between groups were processed by One Way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls test. Results were considered significant at $p<0.05$.

4. Results

4.1 Effects of doxorubicin and melatonin on gene expression

Many studies have shown that melatonin co-administration increases the sensitivity of tumors to inhibition by conventional drugs. In the past few years, many signaling pathways modified by cancer treatments have been described and the modulatory role of melatonin enhancing processes such a cytotoxicity, apoptosis or autophagy induced by chemotherapy have been elucidated.

However, to date, the changes in gene expression induced by chemotherapeutic agents employed at pharmacological concentrations still remain largely unexplored, and the potential effect of melatonin modulating these changes has not been investigated. Therefore, the first goal of this work was to address whether or not melatonin can modulate the changes in gene expression induced by concentrations of doxorubicin equivalent to those administered in therapy for cancer patients.

We used the human breast cancer RT^2 Profiler PCR array containing gene-specific primer sets for 84 breast cancer related genes, in order to characterize the changes that doxorubicin (1µM), either alone, or combined with melatonin (1nM) provoked on the gene expression profile of the estrogen responsive MCF-7 human breast cancer cell line. This array allows the simultaneous analysis of 84 key genes involved in different pathways commonly altered in breast carcinogenesis. The list of genes, relevant to a specific pathway or disease state, include genes participating in one or more processes such as transduction of signals, proteolysis, cell cycle, apoptosis or angiogenesis genes.

When MFC-7 cells were treated with doxorubicin (1µM), the expression of many genes was modified. Also, when doxorubicin was used in combination with a physiological dose of melatonin (1 nM), the pattern of gene expression was modulated, being some of the genes up-regulated and some others down-regulated. Establishing as a criteria a change of at least ± 50% compared to control cells, when doxorubicin was administered alone, we found up-regulation of 11 genes and down-regulation of 28 genes. When doxorubicin was concomitantly administered simultaneously with melatonin, we found up-regulation of 5 genes and down-regulation of 34 genes (Table 4).

The human breast cancer RT^2 Prolifer PCR array contains only one spot for each gene of interest analyzed. That means that we have only one data for each of the conditions tested, so, although the results might be indicative of what occurs after treatment with doxorubicin or doxorubicin plus melatonin, to validate the results obtained for each gene with apparent altered expression, specific qPCRs of the genes identified as down-regulated or up-regulated must be performed.

DOXORUBICIN			DOXORUBICIN+MELATONIN		
Up-regulated	Down-regulated		Up-regulated	Down-regulated	
BCRA1	*AKT1*	*ESR1*	*CDKN1A*	*AKT1*	*MLH1*
CDK2	*AR*	*FOXA*	*MAPK8*	*AR*	*MMP9*
CDKN1A	*BAD*	*GATA3*	*MKI67*	*KRT8*	*MUC1*
KRT18	*BCL2*	*PYCARD*	*PGR*	*BAD*	*MYC*
MYC	*CCNE1*	*TFF3*	*PTEN*	*BCL2*	*PYCARD*
PTEN	*CDH1*	*TGFB1*		*JUN*	*RB1*
RB1	*CTNNB1*	*MAPK1*		*KRT18*	*SLC39A6*
TP53	*CTSD*	*MAPK3*		*MAPK1*	*ESR1*
TWIST1	*IGF1R*	*MAPK8*		*MAPK3*	*FOX1*
VEGFA	*JUN*	*MGMT*		*MGMT*	*GATA3*
XBP1	*KRT18*	*MKI67*		*BRCA1*	*IFGR1*
	MMP9	*MLH1*		*CCNE1*	*TP53*
	PGR	*SLC39A*		*CDH1*	*XBP1*
	THBS1	*MUC1*		*CDK2*	*TFF3*
				CTNNB1	*TGFB1*
				CTSD	*THBS1*
				VEGFA	*TWIST1*

Table 4.1 *Up-regulated and down-regulated genes*

To carry out this purpose, 7 genes were selected for further analysis by specific qPCRs after incubating MCF-7 cells with doxorubicin (1μM) plus melatonin (1nM) for 6 hours. The genes chosen were:

CDKN1A (encoding for p21), that in the gene expression profiler appeared to be up-regulated in both doxorubicin and doxorubicin plus melatonin treatments.

BCL-2, MUC1 and *GATA3*, that in the gene expression profiler appeared to be down-regulated in both doxorubicin and doxorubicin plus melatonin treatments.

TWIST1 is apparently up-regulated by doxorubicin and down-regulated by co-treatment with melatonin.

SERPINE1 and *SNAI2*, genes that did not reach the criteria of a change of at least ± 50% compared to control cells, but seem to be up-regulated by doxorubicin and down-regulated by doxorubicin plus melatonin.

After several amplifications using RNAs isolated from treated MCF-7 cells of at least 3 different experiments, the data obtained for *BCL-2, MUC1, GATA3, SERPINE1 AND SNAI2* qPCRs rendered results no consistent and therefore they were no conclusive. For these genes, further treatments, RNA isolation, and perhaps, the design of

different primers for amplification will be necessary. *CDKN1A* and *TWIST1* are the two genes that gave consistent results in the specific qPCRs.

As shown in figure 8, treatment with doxorubicin significantly enhanced the expression of the tumor suppression gene *CDKN1A* (p21). The *CDKN1A* mRNA levels are five times higher in doxorubicin treated cells in comparison with un-treated control cells. Interestingly, addition of melatonin in combination with this chemotherapeutic agent potentiates this stimulatory effect. The *CDKN1A* mRNA showed a two-fold increase in its levels when cells were treated with doxorubicin plus melatonin, in comparison with only doxorubicin treated cells.

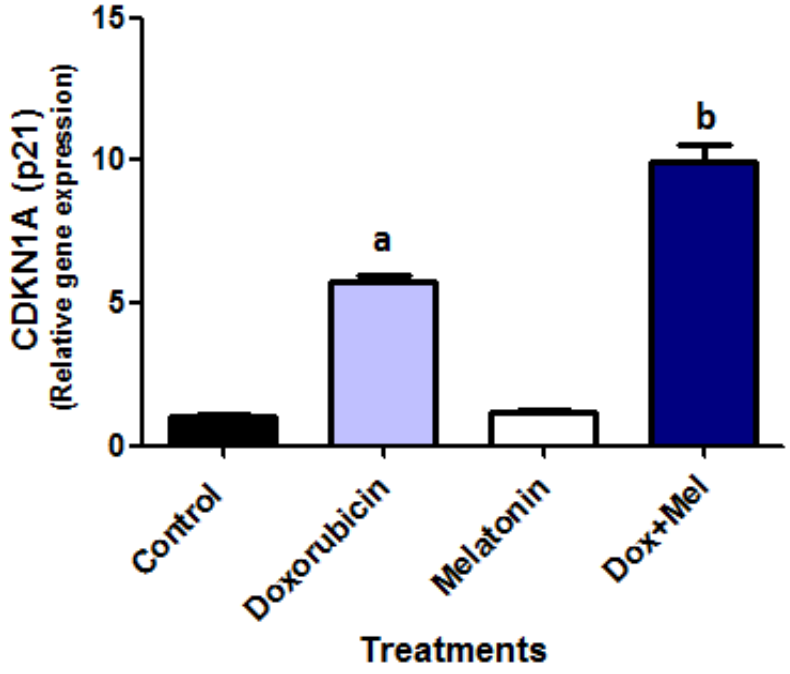

Figure 4. 1. *Effects of doxorubicin and melatonin on CDKN1A (p21) gene expression. Data are expressed as a relative gene expression compared to control cells. (a) p<0.05 versus control and (b) p<0.05 versus doxorubicin.*

The cyclin-dependent kinase inhibitor p21 (also known as $p21^{WAF1/Cip1}$) promotes cell cycle arrest in response to many stimuli. The p21 protein represents a major target of p53 activity and thus is associated with linking DNA damage to cell cycle arrest. It functions as both a sensor and an effector of multiple anti-proliferative signals. Higher levels of expression of *CDKN1A* might be crucial in the execution of apoptosis, one of the mechanisms of defense against de-regulation of the cell cycle machinery, one of the main characteristics of neoplastic transformation. Our results suggest that melatonin and doxorubicin cooperatively act stimulating the expression of p21,

indicating a potential beneficial effect of melatonin when administered together with the anthracycline.

TWIST1 is a transcription factor involved in cancer metastasis. As shown in Figure 9, doxorubicin significantly induced a 15-fold increase in the levels of this factor, in comparison with un-treated control cells. Importantly, when MCF-7 cells were simultaneously treated with doxorubicin and melatonin, the pineal hormone strongly repressed the transcriptional activity of these gene induced by the anthracycline, reaching levels similar to those of control untreated cells.

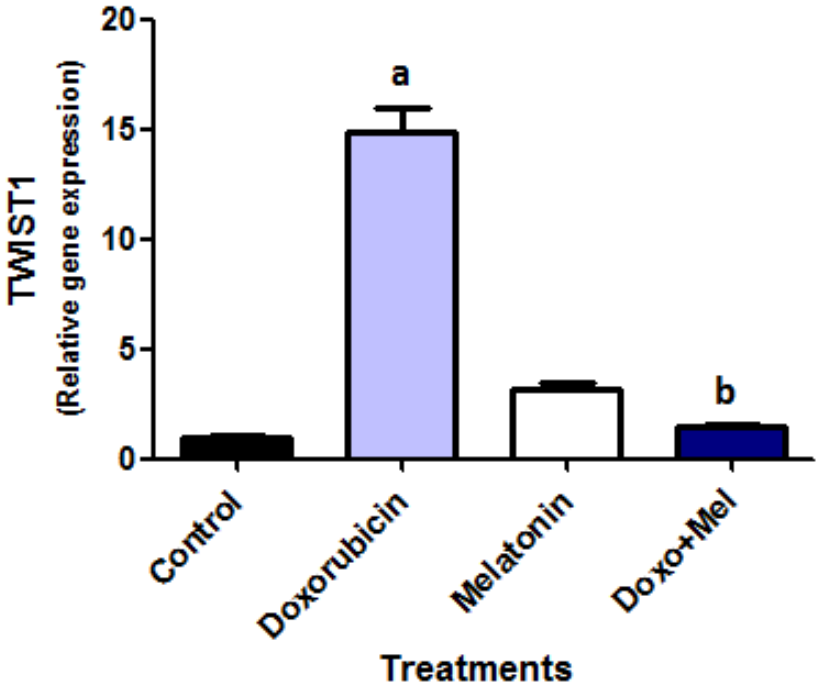

Figure 4.2. *Effects of doxorubicin and melatonin on TWIST1 gene expression. Data are expressed as a relative gene expression compared to control cells. (a) p<0.05 versus control and (b) p<0.05 versus doxorubicin.*

This is an important finding, since TWIST1 is one transcription factor expressed during embryonic development, and silenced in epithelial cells, but reactivated again in multiple carcinomas. When *TWIST1* is expressed in cancer, the probability of invasion and metastasis is higher and the prognosis is poor. Our results strongly suggest that treatment of breast cancer cells with doxorubicin may kill most of the malignant epithelial cells, but at the same time, de-regulated expression of transcription factors such as TWIST1 may occur in the surviving cells. Melatonin, accordingly to our results, might have a protective effect counteracting the undesirable effects of chemotherapeutic drugs.

4.2 Effects of doxorubicin and melatonin on cell proliferation

Once determined that there exist differences in the pattern of gene expression between doxorubicin and doxorubicin plus melatonin treated cells, as mentioned in the objectives of this work, the next point to be investigated was to establish if melatonin potentiated the anti-proliferative effects of doxorubicin in human breast cancer cells. It has been described a synergic effect of high doses of doxorubicin and melatonin on apoptosis on MCF-7 cells, but to date, no research has focused on the effect of the pineal hormone in combination with low doses of the anthracycline.

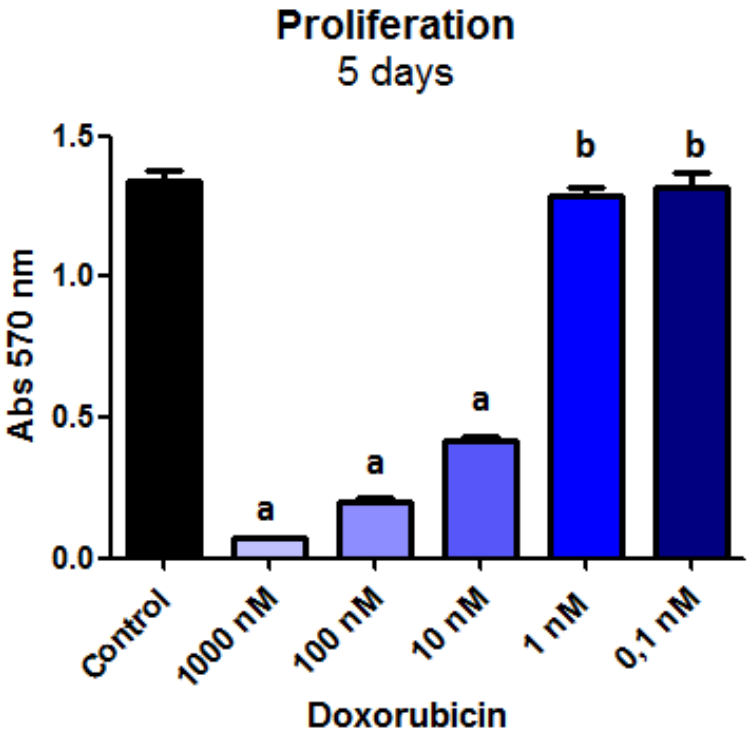

Figure 4.3. *Effects of Doxorubicin on MCF-7 cells proliferation. Date are expressed as relative proliferation compared to the control group (mean±SEM) (a) p<0.001 versus control; (b) p>0.05 versus control.*

In first place, we tested the effect of different concentrations of doxorubicin on MCF-7 cell proliferation. As seen in Figure 10, the anthracycline, at doses comparable to those used in clinical practice (1 μM), inhibited the cell growth in more than a 90%. The anthracycline inhibited the human breast cancer cells proliferation in a dose dependent manner. Doxorubicin (1 nM), or lower concentrations, had no anti-proliferative effects on this cell line.

The results of MCF-7 growing cells treated with doxorubicin and melatonin are shown in figure 11. At physiological concentrations (1nM), melatonin alone, moderately diminished the growth in MCF-7 cells after 5 days of proliferation. This result is in agreement with previous results described in this cell line. When melatonin was added in combination with doxorubicin, the anti-proliferative effect of the anthracycline was potentiated. At concentrations of 10 nM and 5 nM of doxorubicin, the addition of melatonin resulted in a moderate but significant reduction in cell proliferation. At a concentration of the anthracycline of 1 nM, addition of melatonin seemed to have a moderate effect, but these differences did not reach statistically significance.

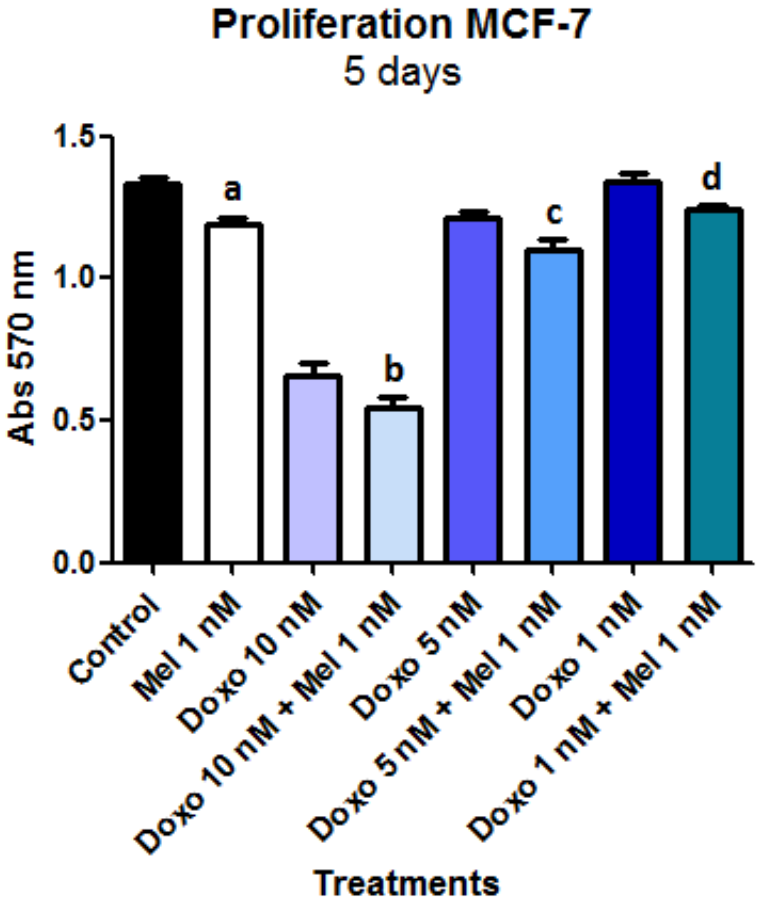

Figure 4. 4. *Potentiation of doxorubicin-induced growth inhibition by melatonin. Data are expressed as relative proliferation compared to the control group (mean±SEM). (a) p<0.01 verus control. (b) p<0.05 versus 10 nM doxorucin dose, (c) p<0.01 versus 5nM dose. (d) p>0.05 versus 1nM doxorubicin dose.*

Our results strongly suggest that combination of melatonin and low doses of doxorubicin might result in a cooperative enhancement of cytotoxicity. These results suggest that one of the advantages of using melatonin as a co-treatment with chemotherapeutic drugs might be to allow the therapeutic doses of anti-cancer drugs

to be reduced, which in turn might help to reduce the side effects and to improve the well-being of the patients.

5. Discussion

Nowadays, breast cancer is the most common cause of cancer death among women worldwide. The most frequent type of breast cancer is ductal carcinoma, originated from cells of the inner lining of milk ducts. Mammary tumors can also been originated in the lobules and adjacent tissues. In many occasions, and due to breast cancer screenings from programs of early detection and prevention, the illness is detected before abnormal cells spread outside the duct. In this case, the cancer is called ductal carcinoma. When the origin is in the lobules, it is known as lobular carcinoma. When the tumor breaks the walls of the glands where they originated and have spread to surrounding tissues it is denominated invasive or infiltrating breast cancer. Finally, another possibility is that cancer cells block the lymph nodes and vessels in the skin causing swelling and redness of the breast, in which case, it is classified as inflammatory breast cancer.

During her lifetime, 1 in 8 women will develop breast cancer. In Spain, it represents one third of all tumors diagnosed among women, with approximately thirty thousand new cases every year. This is a lower incidence in comparison with other countries such as United States, United Kingdom, Netherlands, Italy, France or Germany. The frequency of new cases is slowly growing every year, with a constant increase of 1-2% with an average of 51 cases per 100.000.

Generally, we refer to breast cancer as a single disease. However, it is important to mention that there are at least 21 distinct histological subtypes and 4 different molecular subtypes, associated with distinct risk factors and influencing in treatment options and prognostic. The molecular subtypes are established attending to the presence or absence of progesterone and/or estradiol hormone receptors (HR) and the levels of human epidermal growth factor (HER2), a growth promoting protein. The subtypes are [38]:

- Luminal A (HR+/HER2-): About 75% of mammary tumors express the estrogen receptor (ER+) and/or the progesterone receptor but not HER2. These tumors tend to grow slower and to be less aggressive than other subtypes. They have a most favorable prognosis and they usually respond well to hormonal therapy.
- Luminal B (HR+/HER2+): They also have hormone receptors, but are highly positive for Ki67 (indicating a high ratio of dividing cells) and HER2. They represent about a 10% of breast tumors, and tend to be higher grade and more aggressive.
- Triple negative (HR-/HER2-): About 12%. As curiosity, they are much more common in black women and in premenopausal women with a BRCA1 mutation. They have a poorer short-term prognosis because there is not currently targeted therapy against them.

- HER2-enriched (HR-/HER2+): About 4% of mammary tumors produce an excess of HER2 but they do not express hormone receptors. They are the ones that spread more aggressively and have poorer prognosis than ER+ tumors. However, many targeted therapies (most of them based on monoclonal antibodies) have been developed recently and are contributing to reverse the adverse prognosis.

As said above, most of breast tumors, about an 85%, are hormone receptor positive. The list of risk factors for breast cancer is quite extensive, and includes modifiable and non-modifiable risk factors, which influence the origin and development of breast cancer. Many of the risk factors are linked to estrogens, and depend on the estrogen receptor status of the malignant cells. Several epidemiological and clinical trials link sustained and cumulative exposure to estrogens with increased risk of developing breast cancer. The estrogen carcinogenicity is due to its specific nuclear receptor alpha (ERα) that exerts a potent stimulus on breast cell proliferation enhancing the production of growth factors. The expression, protein levels and activity of this receptor is increased in hormone receptor positive breast tumors. When estradiol binds to ERα, the complex E_2-ERα acts as a transcriptional activator, inducing the expression of genes implicated in cellular proliferation and differentiation in normal breast tissue, but in tumor malignant cells, leads to abnormal gene expression ultimately leading to uncontrolled growth. It seems that, in cancer cells, several proteins interact, bind and protect the estrogen receptor from degradation, leading to enhanced estrogen signaling [39].

The estrogen receptor status is an important fact when deciding the appropriate treatment for breast cancer patients. If the mammary tumor is estrogen receptor positive, the pharmacological agents employed pursue to neutralize the actions of the estrogens on the breast. Hormonal therapy is indicated for ER+ patients**,** pretending to block the transcriptional activation induced by estradiol. These drugs are called selective estrogen receptor modulators (SERMs). They are ER ligands that act as antagonists preventing the estrogen binding to its receptor in cancer cells, and as agonists maintaining the beneficial effects in other tissues like cardiovascular or bones. The most commonly currently employed SERMs are tamoxifen and fulvestrant. They both are competitive inhibitors of estradiol, binding to the estrogen receptor and provoking conformational changes distinct of those triggered by estrogens, in such way that, instead of transcriptional activation, a strong repression of transcription is achieved. In general, tamoxifen is indicated for premenopausal patients, whereas fulvestrant is administered to postmenopausal women. The advantage of this last one is that it does not have agonistic undesirable actions in other tissues (as tamoxifen does). Thus, tamoxifen, but not fulvestrant, increases the risk of developing endometrium cancer [40].

Another choice when treating breast cancer patients is the use of selective estrogen enzyme modulators (SEEMs), drugs that inhibit the enzymes involved in the steroid synthesis or activate the enzymes that inhibit estrogens. Among these medications, we can mention formestane and anastrozole. They have in common its target, the enzyme complex aromatase that converts androgenic precursors into estradiol in malignant cells and tumor surrounding tissues. Indeed, the aromatase activity is increased in

breast cancer cells than in normal tissues, fact that otherwise contributes to support the hypothesis that an increased production of estrogens within breast tumors potentiates their uncontrolled growth, especially in postmenopausal women [41].

Melatonin, the main product of the pineal gland, mitigates cancer at the initiation, progression and metastasis stages. It has been well documented that this indole-amine can inhibit the growth of different types of cancer, but most of initial studies have been performed in estrogen responsive mammary tumors, in experiments conducted *in vivo* in rodents or carried out *in vitro* in human breast cancer cells, mainly in MCF-7 cells, derived from breast adenocarcinoma [25, 42]. It has been reported that melatonin, at physiological concentrations, is a modulator of the E_2-ERα signaling pathway, acting at the same time both as a SERM and as a SEEM. Melatonin decreases the protein levels of ERα; in parallel, the pineal hormone destabilizes the E_2-ERα complex, interfering with its binding to estrogen responsive promoters, probably by inducing conformational changes in E_2-ERα-calmodulin complex that leads to inhibition on the estrogen-dependent transcriptional activation by diminishing the ERα expression [27]. The pineal hormone also acts as a SEEM controlling the enzymes involved in the biosynthesis and transformation of estrogens. This action is mainly performed by inhibiting the aromatase activity and its expression on basal conditions, which impairs the synthesis of estrogens from androgenic precursors. Furthermore, melatonin is involved in the regulation of the enzymes involved in the biosynthesis of estradiol in both epithelial malignant cells and surrounding tissues, mainly in adipocytes and endothelial cells. Apart from aromatase, the pineal hormone reduces the activity and expression of sulfatase, and 17-beta-hydroxyesteroid dehydrogenase, involved in the activation of estrogens, and stimulates the transcription and activity of estrogen sulfotransferase, involved in the inactivation of circulating estrogens [25].

In the last 5 years, multiple clinical trials have shown that melatonin, when simultaneously administered with established chemotherapy protocols, enhances the effects of chemotherapeutic drugs in different types of cancer. It seems that melatonin co-administration improves the sensitivity of cancer cells to conventional cancer treatments. Thus, melatonin inhibits migration, invasion and spreading of cancer cells by limiting its entrance into the vascular and lymphatic systems, preventing metastasis at distant sites. Remarkably, many studies demonstrate the capability of melatonin in reducing the toxic consequences of anticancer drugs while increasing its efficacy; and some recent reports describe how melatonin renders cancers, previously characterized as resistant to treatment, to chemotherapeutic drugs sensitive to these same therapies [43].

For example, melatonin induced a decline in the frequency of chemotherapy induced asthenia, cardiotoxicity and neurotoxicity, as well as an increase in the in the 1-year survival rate and the objective tumor regression in patients with metastatic solid tumors with poor clinical status. After 5 years, the regression rate and survival was significantly higher in patients treated with melatonin [32, 35].

Recently, many groups have focused their attention to the potential benefits that melatonin presence could have to improve the effects of chemotherapy, or by contrary, how melatonin absence can lead to reduced efficacy of chemotherapeutic

drugs. When the effect of melatonin on doxorubicin treatment was tested, it has been described how a circadian disruption of nocturnal melatonin production contributes to a complete loss of tumor sensitivity to doxorubicin chemotherapy [36]. Melatonin co-treatment increases doxorubicin intracellular concentrations in cancer cells, contributing to sensitize them to the chemotherapy agent, suggesting a downregulation of the P-glycoprotein, a pump involved in the efflux of toxic molecules out of the cells [44]. Consistently with these findings, co-treatment with melatonin and each of three different chemotherapy agents (5-fluorouracil, cisplatin and doxorubicin) resulted in melatonin enhanced chemotherapy-induced cytotoxicity and apoptosis in the rat pancreatic tumor cell line AR42J[34]. In ER-responsive breast cancer induced in rats subsequently treated with doxorubicin, melatonin co-treatment resulted in lighter tumor weights, increased tumor cell apoptosis, higher expression of E-cadherin and higher survival rate. In MCF-7 cells, melatonin had synergic effects with doxorubicin on apoptosis and activation of transient receptor potential vanilloid (TRPV1) channels [37].

As said above, it has been described that melatonin influences the effects of chemotherapeutic drugs in several cancer models tested. Thus, melatonin increases doxorubicin concentrations in cancer cells, sensitizing them to the anthracycline. Melatonin also enhances cisplatin-induced cytotoxicity and apoptosis in lung cancer cells and tunicamycin triggered apoptosis in breast cancer cells [45, 46].

We have studied in this work the effects of combinations of melatonin and doxorubicin, a cytotoxic anthracycline antibiotic used as a chemotherapeutic drug in breast cancer treatments. Anthracyclines are known to intercalate into DNA *in vitro*, causing DNA breaks and inhibiting both DNA and RNA polymerases, impairing DNA replication and transcription. Anthracyclines also generate free radicals causing more DNA damage. The effects of melatonin on doxorubicin cytotoxicity at low concentrations of the anthracycline and the modulatory actions of the pineal hormone on the gene expression changes induced by doxorubicin have not been tested to date.

Like in the above cited studies [44-46], our results showed that melatonin enhanced doxorubicin-induced cytotoxicity. We performed our investigation in estrogen dependent MCF-7 breast cancer cells where first we established that doxorubicin stopped cell growth in a dose dependent manner. To test the effect of the pineal hormone on the anti-proliferative actions of the anthracycline we chose the concentrations of doxorubicin of 1 nM, 5nM and 10 nM. We discarded higher doses of doxorubicin (such as concentrations of 1 μM, equivalent to pharmacological doses used in clinic) since doxorubicin at these high concentrations effectively killed MCF-7 cells in more than 90% in just 5 days, and longer times of exposure to the anthracycline resulted in non-survival of the cells. Our results also showed that using melatonin in a dose of 1nM, the pineal hormone has been effective in arresting the cell proliferation. In addition, melatonin significantly enhanced the cytotoxic effects of low doses of doxorubicin (10 nM and 5 nM). At a concentration of 1 nM of doxorubicin, the simultaneous addition of melatonin resulted in a moderate inhibitory effect that did not reach statistically significance.

These results with low concentrations of the anthracycline are very interesting since the dose of melatonin used was 1 nM, similar to nocturnal physiological concentrations of the pineal hormone in plasma, and melatonin does not have any adverse effect recognized at this or even higher concentrations. Additionally, our results point to melatonin as an enhancer of doxorubicin anti-proliferative effects when the anthracycline is used at sub-pharmacological doses. These results strongly suggest that combinations of melatonin and chemotherapy drugs should be taken into consideration, since they might have potential applications. If the same enhancement of the cytotoxicity is obtained in clinical tests, patients receiving doxorubicin might benefit in the future of a co-treatment with melatonin as an adjuvant agent, allowing a reduction in the amount of the chemotherapeutic drug employed, which could result in better tolerance, less undesirable side effects and higher efficacy.

Results from several clinical trials, many experiments performed in animal models and also performed in cancer cell lines, point to melatonin as a promising agent resulting in improvement in tumor remission, survival and ameliorating the side effects of chemotherapy. However, there is very little information about what changes in gene expression are triggered in the malignant cells in response to chemotherapy, and how melatonin can modulate those changes. There are a great amount of genes known to be altered in hormone responsive breast cancer, encoding for proteins participating in signaling transduction, angiogenesis, adhesion, apoptosis, control of the cell cycle or elimination of toxic compounds out of the cells. One of the objectives of this work was to test the expression profile of the estrogen responsive MCF-7 cell line, in response to doxorubicin and a combination of doxorubicin plus melatonin.

We selected for the analysis the human breast cancer RT^2 Profiler PCR array (Qiagen, USA), that contains gene-specific primer sets for 84 genes involved in breast cancer. We intended to characterize the changes that doxorubicin at doses equivalent to those used in patients (1µM), might have on the gene expression profile of the MCF-7 cells.

When MFC-7 cells were exposed to doxorubicin (1µM), the expression of many genes was altered. Also, when doxorubicin was combined with a physiological dose of melatonin (1 nM), the pattern of gene expression was modified. Establishing as criteria a change of at least ± 50% compared to control cells, we found that doxorubicin up-regulated 11 genes and down-regulated 28 genes, related to untreated control cells. When doxorubicin was added simultaneously with melatonin, we found up-regulation of 5 genes and down-regulation of 34 genes.

Since the human breast cancer RT^2 Prolifer PCR array contains only one spot for each gene of interest, it is necessary to validate the results, performing specific qPCRs of the genes identified as down-regulated or up-regulated. The genes chosen for further specific analysis were *CDKNA1* (p21), *MUC1, GATA3, SERPINE1, SNAI2, TWIST1* and *BCL2*. Unfortunately, we did not obtain enough reproducible data from *MUC1, GATA3, SNAI2, SERPINE1* and *BCL2*. More specific PCRs should be performed, and perhaps the design of new primers will be necessary.

However, we obtained reproducible and interesting results for *CDKNA1* (p21), and *TWIST1*. Melatonin cooperatively enhanced the effect of doxorubicin inducing the

expression of p21. By contrary, doxorubicin strongly induced the expression of *TWIST1*, whereas melatonin counteracted this effect.

CDKN1A (cyclin dependent kinase inhibitor 1A), also known as p21 or else $p21^{WAF1/Cip1}$, is a cyclin-dependent kinase inhibitor that has the ability to inhibit cell cycle progression at G1 due to its capacity to inhibit the activity of cyclin-dependent kinases (CDKs)-cyclin complexes. A unique feature of p21 that distinguishes it from the other cyclin-dependent kinase (CDK) inhibitors is its ability to associate with the proliferating cell nuclear antigen (PCNA), an auxiliary factor for DNA polymerases δ and ε. P21 is regulated by multiple signals, but the main transcriptional regulator of p21 is p53, since the *CDKN1A* gene contains two p53 responsive elements in its promoter. Stress signals, such as DNA damage induced by chemotherapeutic agents, or oxidative stress up-regulate p53 activity, which subsequently results in up-regulation of p21 expression. Recently, it has become apparent that many pathways independent of p53 stimulate p21 too. For example, the breast cancer susceptibility gene 1 (BRCA1), or TGF-β induce the expression of this cyclin inhibitor, whereas the family of c-Myc factors repress its transcription [47].

When p21 acts as an inhibitor of cell cycle progression, the suppressor activity is developed by inducing growth arrest, differentiation and senescence. As said earlier, a variety of stimuli like DNA damage, oxidative stress or cytokines, are sensed for p21 which responds promoting growth-inhibitory actions that depends on its ability to inhibit cyclin-dependent kinase 2 (*CDKNA2*) and cyclin-dependent kinase 1 (*CDKNA1*). P21 can also inhibit cellular proliferation by inhibiting *PCNA*, which is required for S phase progression [48, 49].

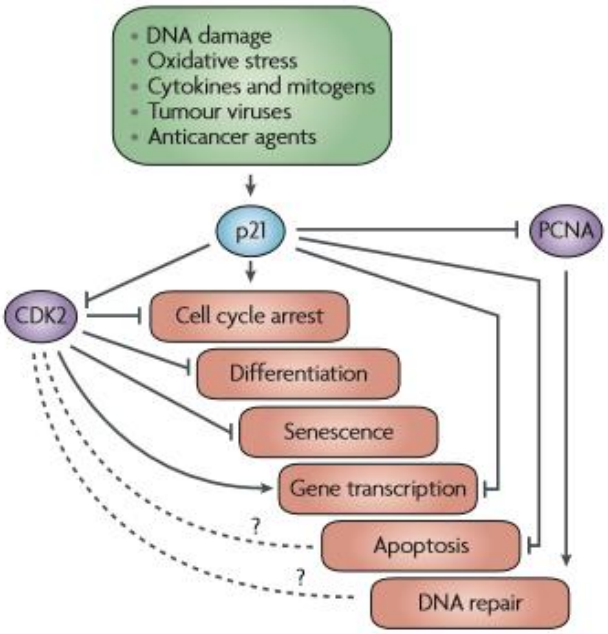

***Figure 5.1**. Central role of p21 in sensing and responding to a plethora of stimuli. (Taken from Nature review, volume 9. June 2009)*

When we analyzed the expression of p21 by specific qPCRs, we found that in doxorubicin treated cells the expression of p21 was strongly stimulated by the anthracycline. Interestingly, when melatonin was added in combination with doxorubicin, p21 gene expression was further stimulated. Induction of p53 and $p21^{WAF1/Cip1}$ by melatonin has been previously proposed as part of the mechanism by which melatonin inhibits proliferation of breast cancer cells [50]. Our results point to a beneficial effect of melatonin when administered together with the anthracycline. Thus, the stimulation of p21, linked to cell cycle arrest and apoptosis is beneficial for the treatment of breast cancer, and we demonstrated a cooperative effect in its expression when melatonin was added in combination with the chemotherapeutic treatment.

The second gene analyzed in this work was *TWIST1*. The TWIST1 proteins are basic helix-loop-helix (bHLH) transcription factors implicated in cell lineage determination and differentiation; they are essential for mesoderm specification and differentiation in embryonic development. During embryogenesis, its lack displays multiple defects including failure on neural tube closure or an increase at the apoptosis of the somites.

After birth, expression of this gene is mainly restricted to some precursor cells such as myogenic, osteoblastic or chondroblastic, maintaining their undifferentiated state. Twist proteins were recently found to behave as a key regulator of adaptative thermogenesis and to perform important roles in lymphocyte function and maturation. In healthy adult tissues, TWIST1 expression remains largely repressed. Invariably, TWIST proteins are undetectable in epithelial cells [51].

By contrary, TWIST1 gene expression is frequently reactivated in multiple carcinomas (including breast, bladder or lung among others), melanomas and sarcomas. Its expression in all these cancer types is associated with high grade, invasive and metastatic lesions that leads to a poor prognosis. As said above, TWIST1 gene expression is silenced after birth, but its reactivation is based in deregulation of the pathways that control development during embryogenesis. For example, stress conditions such as hypoxia or mechanical constrains turn on TWIST1 expression in physiological conditions and in pathological conditions too. TWIST1 is activated by several signaling pathways, such as Wnt, NF-κB, c-Myc [52], some of which have been previously reported as down-regulated by melatonin.

TWIST proteins have also oncogenic and pro-metastatic properties; they prevent senescence and apoptosis, and promote invasion and metastasis dissemination in multiple cancer types including breast, liver or prostate. This potential relies in their ability to induce an epithelial to a mesenchymal transition (EMT) converting adjacent, joined and polarized cells into motile and isolated cells, capable to migrate through the basement membrane and to infiltrate into the surrounding extracellular matrix. The EMT is promoted by upregulation of the expression of mesenchymal markers such as N-cadherin, and turning-down the expression of epithelial specific proteins such as E-cadherin [53].

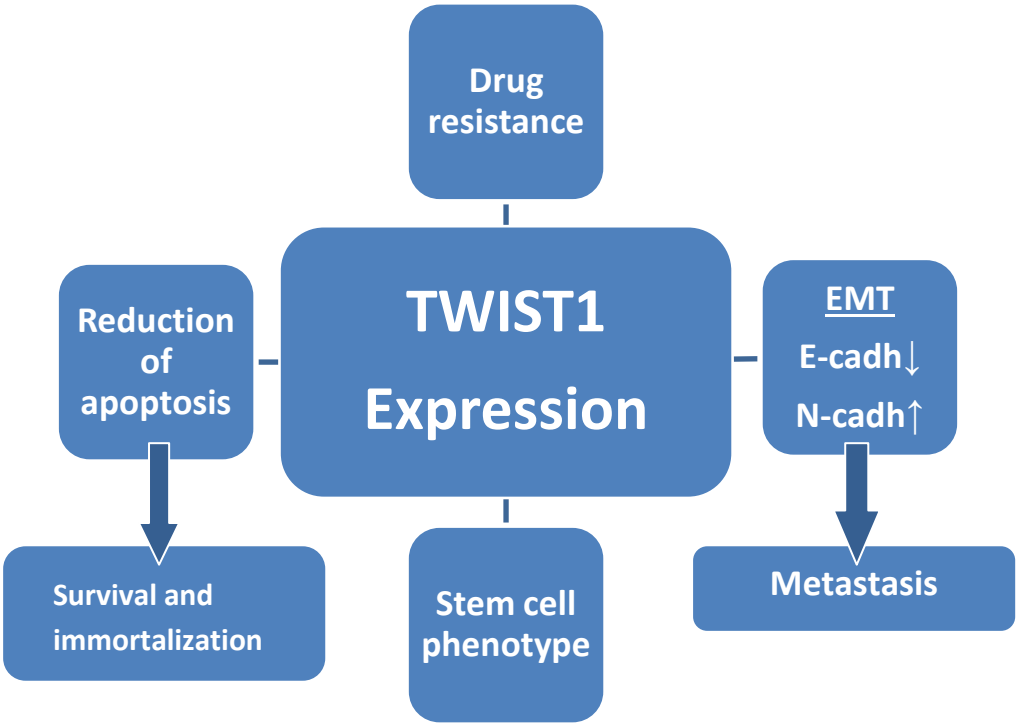

Figure 5.2. *Actions unleashed by TWIST1 expression*

Our results showed that *TWIST1* transcription was induced 15-fold by doxorubicin, whereas it was strongly repressed when MCF-7 cells were simultaneously treated with doxorubicin and melatonin, reaching levels similar to the control. Therefore, it seems that melatonin co-treatment with doxorubicin decreases the expression of TWIST1 proteins in epithelial malignant cells. In terms of breast cancer patients treated with doxorubicin, our results suggest that perhaps those cancerous cells who survived the anthracycline may be undergoing reactivated expression of genes with pro-metastatic properties such as *TWIST1*. The repression of this protein by melatonin might also prevent cells that have survived to the effects of chemotherapy from performing the mesenchymal transformation of them that could lead to a new metastasis. [51, 54]

It has been described that some chemotherapeutic drugs promote epithelial-mesenchymal transition. In MCF-7 cells, doxorubicin has been reported to inhibit the microRNA miR-448 that is inversely correlated with NF-κB and TWIST1 activation. In gastric cancer cells, the long term effects of doxorubicin treatment on EMT markers have been addressed. Human gastric BGC-823 cells were treated with the anthracycline. Most of the cells died after a week, but the surviving cells formed colonies. These long term survival cells expressed high levels of EMT molecules such as vimentin and *TWIST1* [55].

Recently, some reports have related *TWIST1* and p21 levels in response to stress signals. Specifically, *TWIST1* attenuates p53 mediated responses in cancer cells, by suppressing the transcription of $p19^{ARF}$ (which is a p53 activator) and the p53 target $p21^{WAF1}$. Moreover, *TWIST1* represses the transcription of $p16^{INK4A}$ to allow cancer cells

to escape RB-mediated cell cycle control [56]. Additionally, *TWIST1* also directly interacts with p53 to inhibit its post-translational modification and to facilitate its MDM2-mediated degradation [57]. Therefore, *TWIST1* simultaneously affects p53 and RB signaling pathways, and in the first case, at least partially, its effects are mediated by $p21^{WAF1}$.

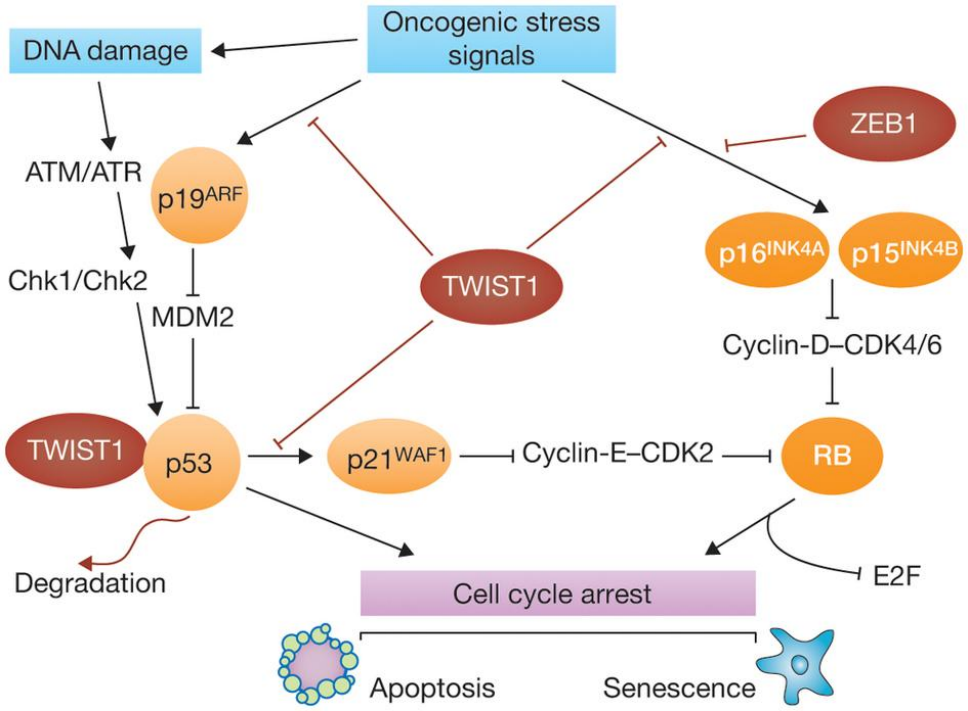

Figure 5.3. TWIST1 and p21 levels related in response to stress signals. (*Taken from Alain Puisieux et al. Oncogenic roles of EMT-inducing transcription factors, 2014)*

In summary, our results proved that melatonin was capable to further enhance the $p21^{WAF1}$ expression induced by doxorubicin whereas, at the same time, counteracted the stimulated transcription of *TWIST1* in response to the anthracycline. Therefore, melatonin might have a beneficial effect when used as an adjuvant treatment with conventional chemotherapeutic drugs such as doxorubicin since it counteracts the anthracycline undesirable effects whereas it potentiates its cytotoxic actions.

6. Conclusions

- Doxorubicin induces changes in gene expression of transcription factors, adhesion molecules, tumor suppressor genes, pro-apoptotic and anti-apoptotic genes in MCF-7 cells. Those changes are modulated by melatonin, accordingly to its previous well established anti-tumor and oncostatic actions.

- Melatonin acts cooperatively with doxorubicin enhancing the expression of p21.

- Addition of melatonin revert the stimulatory effect of doxorubicin on TWIST1 expression.

- The MCF-7 cell proliferation is inhibited in a dose dependent manner by doxorubicin. When melatonin is added as a co-treatment, the anti-proliferative effects of the anthracycline drug are enhanced.

- In summary, the effects of the joint treatment of doxorubicin and melatonin showed to be beneficial both improving the anthracycline effects inhibiting MCF-7 cell proliferation and regulating p21 and TWIST1 expression in breast cancer cells.

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I would like to dedicate this work to my parents and my sister, who have been by my side these years encouraging and believing in me, and to my boyfriend for helping me with technologies.

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