Sensitizing effects of melatonin on docetaxel treatment in MCF-7 breast cancer cells

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1.- Background

1.1.- Breast cancer

1.1.1.- Epidemiology

According to the World Cancer Research Fund International, breast cancer is the most common cancer in women worldwide, with nearly 1.7 million new cases diagnosed only in 2012. About 1 in 8 women will develop breast cancer over the course of her lifetime. The American Cancer Society´s estimates for breast cancer in the United States for 2015 are about 231,840 new cases of invasive breast cancer, 60,290 new cases of carcinoma in situ and about 40,290 deaths. Besides that, breast cancer is the second leading cause of cancer death in women only exceeded by lung cancer. Deaths rates have been declining since 1989, especially in women under 50, probably the result of an earlier detection as well as improved treatments (**Figure 1.1**). One fact that might also explain this decrease is the reduced use of hormone replacement therapy (HRT), after the results of the study called the Women´s Health Initiative published in 2002, which suggest a connection between HRT and increased breast cancer risk.

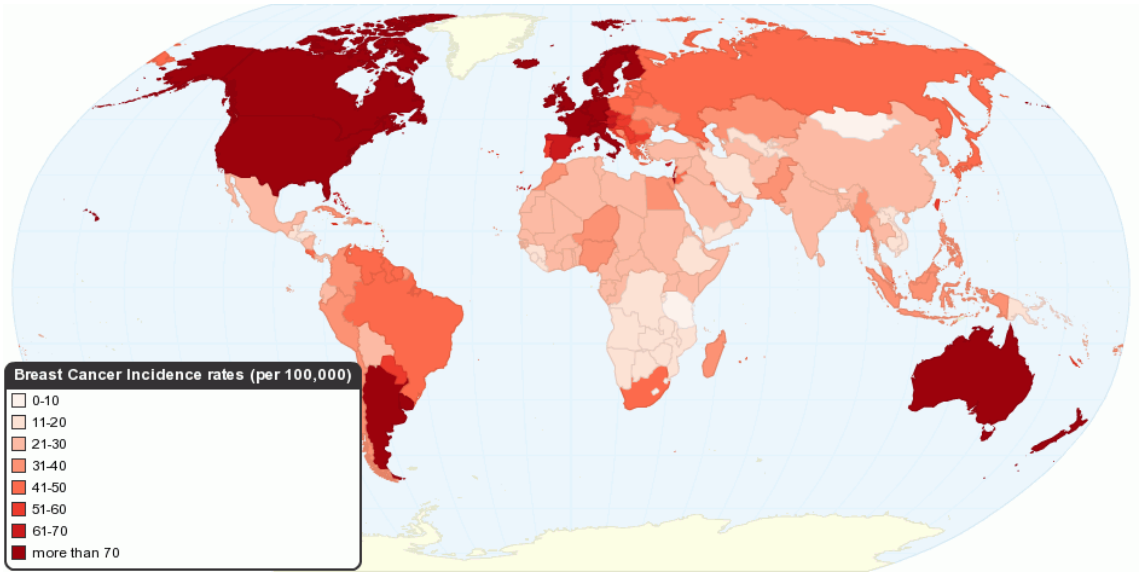

Figure 1.1: Incidence of breast cancer worldwide. New breast cancer cases annually per 100.000 women.

In Spain, breast cancer is the leading cause of cancer mortality in women with 6,075 deaths in 2012 and with an annual incidence of over 25,000 new cases as publish in the report GLOBOCAN (globocan report, *http://globocan.iarc.fr*, 2012).

1.1.2.- Etiology

Breast cancer can be defined as a group of diseases due to various alterations (like mutations, deletions or translocations) in the DNA of mammary epithelial cells that cause an uncontrolled and autonomous proliferation of those cells. Thus a growing tumor, which is capable of invading adjacent tissues and spreading to other parts of the body, by the spread of tumor cells through the lymphatic system and / or blood vessels, producing metastasis in tissues removed from the forms primary focus, is formed. Alterations in DNA that initiate tumorigenesis can be somatic or germ and occur both in the ductal cells ducts (ductal carcinoma) and lobules (lobular carcinoma). There are many parameters influencing the origin and development of breast cancer. These factors can be classified as modifiable and non-modifiable risk factors (**Figure 1.2**):

Non-modifiable Risk Factors	Modifiable Risk Factors
Gender and age	Body weight
Personal cancer history	Physical activity
Family cancer history and genetics	Alcohol use
Early menstruation and late menopause	Smoking
Breast density	Exposure to hormones: the Pill, IVF, and HRT
Breast conditions	Pregnancy and breastfeeding
	Radiation exposure

Figure 1.2: Breast Cancer Risk Factors.

- Being female: women are much more likely than men to develop breast cancer (it affects women in a ratio of 99:1 compared to men).

- Aging: Age is an established risk factor for breast cancer. The older a woman is, the more likely she is to get breast cancer. Rates of this disease are low on women under 40 and begin to increase after this age, being highest in women over age 70.

- Race: This type of cancer affects white or Caucasian women in a greater proportion than women of African or Asian descent.

- The reproductive years: Menarche and menopause mark the onset and cessation of ovary activity. Breast cancer risk increases by a factor of 5% for every year younger at menarche and a

factor of 3% for every year older at menopause. This is related to the time span of exposition to estrogens, the ovarian hormones that play a key role in the normal mammary gland development but which in turn can be considered as carcinogens as mentioned later.

- Body Mass Index: It is a measure of fat based on weight and height; the risk of breast cancer is directly related to height. Thus, the risk increases by 10% for every 10 cm of increase in height. Obesity also correlates with an increased risk, especially in postmenopausal women.

- Genetic factors: *BRCA1* and *BRCA2* are tumor suppressor genes that help to repair damaged DNA; when either of these genes is mutated, DNA is not repaired properly. Together, specific inherited mutations in *BRCA1* and *BRCA2* account for about 20 percent of hereditary breast cancers and about 10 percent of all breast cancers.

- Habits: Unhealthy habits such as high-fat foods, alcohol, lack of physical activity and tobacco (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004) are risk factors. In addition, disruption of circadian rhythms causes dramatically affect the pattern of synthesis and secretion of hormones such as melatonin, thus promoting the development of tumors. In a study comprising more than 100,000 women, the main conclusion was that those who worked more than 20 years in night shifts have a much higher risk of developing the disease (**1**).

- Hormone replacement therapy (HRT): HRT usually combines estrogen and progesterone. Many postmenopausal women took HRT for many years to ease menopausal symptoms (hot flashes, fatigue). The combined use of estrogen and progesterone increases the risk of developing mammary tumors.

- Environmental factors: They include things found in nature that we eat, drink, touch or breathe; they can be physical, such as ionizing radiation or exposure to electromagnetic fields, or chemicals such as organic solvents or metals. Usually they are not considered carcinogenic but its effects are cumulative and long-term exposure can cause hormonal disturbances leading to cancer.

From the physiological point of view, one of the most interesting factors to be considered is the time span of exposure to estrogens. Increased exposure to natural or synthetic estrogen results in a higher risk of developing a breast tumor.

1.1.3.- Role of estrogens and hormonal therapy

Since 1896, when George Beatson performed a bilateral oophorectomy that resulted in a remission of a metastatic breast tumor, many studies have shown the relationship between ovarian function and an increased risk of developing a mammary tumor. The main steroid hormone is

estradiol. Estradiol may be considered as carcinogenic since it is capable of inducing neoplastic transformations similarly to other well-known carcinogens in human mammary epithelial cells. Although the ovaries are the organs that are responsible for the main production of estrogen, it can also be synthesized in peripheral tissues from androgens through the action of the enzyme complex aromatase, as it occurs in menopause after cessation of the follicular function. Therefore, ovariectomy is not sufficient to prevent the occurrence or to stop tumor progression. In this situation it is particularly important the adipose tissue that produces estradiol which acts in the mammary gland; It is also very relevant that the breast tissues themselves may increase the local concentration of estrogen by altering the expression of various genes, such as overexpression of aromatase.

There are several hypotheses to explain the carcinogenic mechanisms of estrogen in breast cancer:

1. **Effects mediated by the estrogen receptor:** estradiol binds to its receptor protein that behaves as a transcription factor that stimulates the expression of genes involved in survival, cell proliferation, invasiveness and metastasis. One of the consequences is that the increase in the proliferation rate also favors the occurrence of errors in replication and point mutations.
2. **Genotoxic effects:** estrogen can be metabolized to generate quinone derivatives. These compounds bind with high affinity to adenine and guanine bases in DNA destabilizing its glycosidic bond and causing their elimination by depurination. This results in point mutations that can lead to neoplastic transformations. To support this theory, it has been published that knockout mice for the estrogen receptor are capable of developing tumors when treated with estradiol (**2**).
3. **Induction of aneuploidy:** is the abnormal chromosome segregation during cell division which results in daughter cells with an aberrant number of chromosomes. Estradiol appears to be involved in aneuploidy patterns that appear in the early stages of mammary tumor lesions.

One of the main goals in the treatment of breast cancer is to neutralize the effects of estrogens on the breast. The pharmacological strategies employed to selectively neutralize the effects of estrogens on the breast are:

- The use of SERM (Selective Estrogen Receptor Modulators). The SERMs are substances that bind and block the estrogen receptor (ER), preventing the estrogen binding, thus acting as estrogen antagonists. When a SERM binds to and inactivates ER many physiological responses in the cell are impaired. Examples of such modulators are tamoxifen and fulvestrant. The main problem is that Tamoxifen, behaves as an estrogen agonist in other tissues such as endometrium (generating cellular responses) thereby increasing the probability of generating cancer. Use of

fulvestrant represents a novel endocrine therapy in breast cancer as it competitively binds to ER with high affinity and downregulates its expression, blocking its function and promoting their degradation. Fulvestrant confers benefits over other SERMs, such as tamoxifen, since it does downregulate ER and has no agonistic effects in other tissues. After many trials, use of fulvestrant in postmenopausal women has eventually being approved. Yet another possible advantage of fulvestrant to be considered is that tumors with mutations in the p53 tumor suppressor gene usually develop resistance to tamoxifen, but they do not seem to change in the same way when treated with fulvestrant.

- The use of SEEM (Selective Estrogen Enzyme Modulators). The SEEMs are inhibitors of the enzymes involved in the synthesis of steroid hormones (therefore preventing estrogen synthesis) or activators of enzymes that inhibit estrogens. Two examples of such modulators are formestane and anastrozole, two drugs that act as inhibitors of the enzyme complex aromatase.

1.2.- Melatonin and mammary cancer: *In vitro* and animal studies

Melatonin is an indole hormone produced mainly by the pineal gland. It is a ubiquitously distributed and functionally diverse molecule. Melatonin employs a diverse set of mechanisms to modulate the physiology and molecular biology of cells. Many of its actions are mediated by G-protein coupled melatonin receptors in cellular membranes; other actions seem to involve orphan receptors or molecules such as calmodulin. Additionally, melatonin can detoxify free radicals and related oxygen derivatives via receptor-independent pathways (**3**).

Concerning tumorogenesis, the most common conclusion in animal models is that either experimental manipulations that activate the pineal gland or the administration of melatonin reduces the incidence and development of chemically-induced mammary tumors, whereas pinealectomy usually stimulates breast cancer growth. Epidemiological studies have shown a low incidence of breast tumors in blind women. A moderate increase in breast cancer among women who have worked extended periods of rotating night shifts (light exposure during the night suppresses melatonin production) has also been reported (**1, 4**).

The antiproliferative actions of melatonin on MCF-7 cells have been studied for more than two decades. The data available suggest that the inhibitory action of melatonin on breast cancer cells is based on its interaction with either the synthesis of estrogens or with the estrogen signaling pathway. Thus, the pineal hormone is capable of down-regulating the expression and activity of the enzymes necessary for the synthesis of estrogens from androgenic precursors, therefore behaving as a selective estrogen enzyme modulator (SEEM). In MCF-7 cells, melatonin, at physiological concentrations, has been reported to reduce the activity of aromatase, the key enzyme responsible for estrogen biosynthesis, under basal conditions or when aromatase activity

is stimulated by cAMP or cortisol. When expression of the CYP19 gene (encoding for aromatase) was examined, melatonin down-regulated its expression at the transcripitional level (**5, 6**). The major bloodstream circulating form of estrogen is estrone sulfate, a biologically inactive form of estrogen, which acts as an estrogen reserve. The enzyme steroid sulfatase (STS) converts inactive estrogen sulfates to estrone and estradiol. Estrone can be further reduced to the biologically active estrogen by 17β-hydroxysteroid dehydrogenase type 1 (17β-HSD1). Finally, the estrogen sulfotransferase (EST) sulfonates estrogens to form biologically inactive estrogen sulfates. STS and EST are considered to be involved in the regulation of in situ levels in hormone-dependent tumors; Melatonin modulates the expression and activity of aromatase, STS, 17β-HSD1 and EST not only in tumor cells, but also in surrounding cells such as fibroblasts and endothelial cells (**7, 8**).

Melatonin can also counteract the effects of estrogens, thus behaving as a naturally occurring selective estrogen receptor modulator (SERM) (**Figure 1.3**). The mechanisms involved in the antiestrogenic actions of melatonin are still being elucidated. Unlike other antiestrogens such as tamoxifen, melatonin does not directly bind to the estrogen receptor. In breast cancer cell lines, melatonin decrease the expression of ERα and impairs the estrogen-dependent transcriptional activation through destabilization of the E2-ER complex from binding to DNA in both estrogen response element and AP1 containing promoters. This effect seems to be 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) (**9**). Interestingly, melatonin does not affect the binding of coactivators to ERα indicating that melatonin action is different from that of other antiestrogens used in breast cancer therapy. Interestingly, only ERα but not ERβ interacts with calmodulin (**10**). Substitution of lysine residues 302 and 303 of ERα for glycine rendered a mutant ERα unable to bind calmodulin, and that becomes insensitive to melatonin (**9**). The effects of melatonin might also been explained in terms of its binding to its specific membrane receptors resulting in an interplay with the ER- signaling pathway (MT1). The MT1 receptors are present in human breast normal tissues, but also in tumoral tissue. A link between both signaling pathways (melatonin and estradiol) could be the opposite effects that they cause over cAMP intracellular concentrations.

In breast cancer cells, estrogens activate adenylate cyclase increasing cAMP levels in a short-time mechanism independent of transcription. The increase in cAMP levels synergizes with the genomic actions of estradiol enhancing ER-mediated transcriptional activation. By contrary, melatonin through its binding to its membrane receptor MT1, inhibits the adenylate cyclase resulting in decreased cAMP levels (**11**).

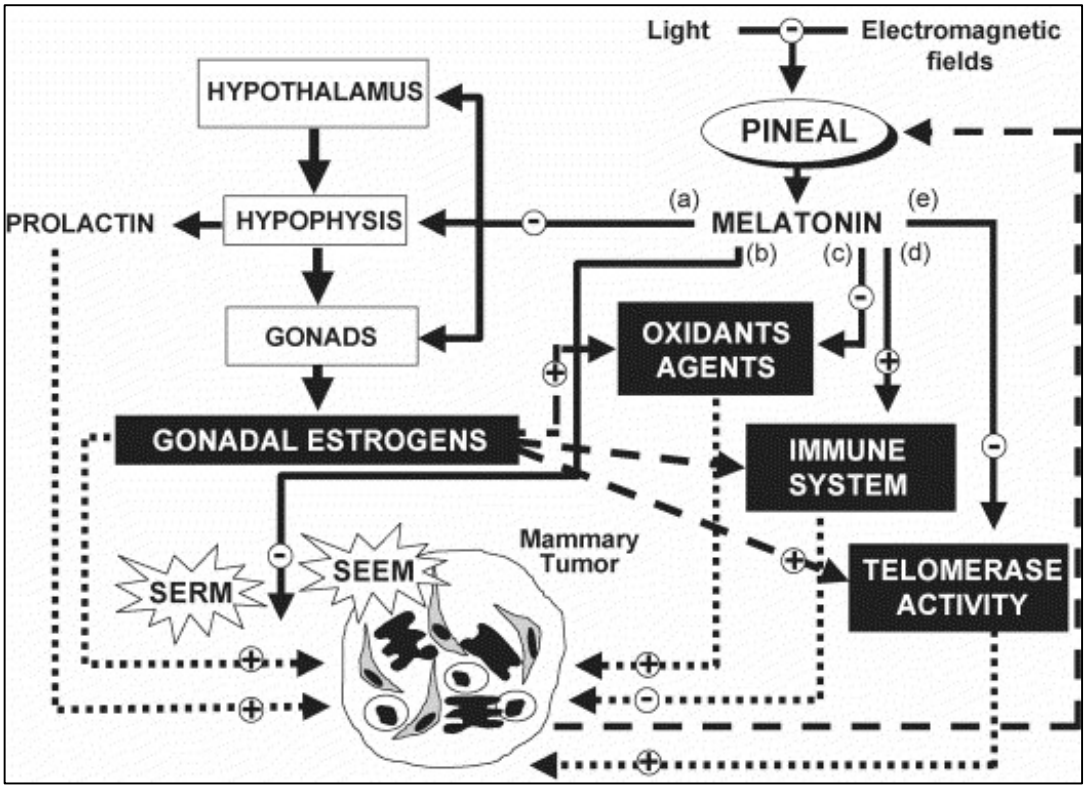

Figure 1.3: Diagram that shows the mechanisms through which melatonin may modulate mammary carcinogenesis: **(a)** indirect effects on the neuroendocrine reproductive axis; **(b)** anti-estrogenic actions on the breast cancer cells, melatonin thus behaving as a selective estrogen receptor modulator (SERM) and a selective estrogen enzyme modulator (SEEM); **(c)** anti-oxidative effects; **(d)** immunomodulatory actions; or **(e)** inhibition of telomerase activity.

The fact that only human breast cancer cell lines expressing estrogen receptors have been described as responsive to the antimitogenic effects of melatonin strengthen the hypothesis that melatonin´s oncostatic actions are mediated via its effects on the tumor cells estrogen-response pathway. Melatonin is able to block, under different culture conditions, the mitogenic effects of estradiol. The antiproliferative effect of this indoleamine could be explained through the modulation of estrogen-regulated proteins, growth factors and proto-oncogenes (TGFβ, c-MYC, PS2, progesterone receptor (PGR) and c-fox) in human breast cancer cells. Estradiol stimulates cell proliferation and induces cell cycle progression; the antiproliferative effect of melatonin (like tamoxifen) is cell-cycle specific by causing a G_1-S transition delay. Changes in cell-cycle kinetics typically involve changes in some regulatory proteins of the cell cycle. The modulatory effect of

melatonin on the cell cycle can be explained by its effects on the expression of some of the proteins involved in the control of the G_1-S transition. Thus, it has been demonstrated in breast cancer cells that melatonin increases the expression of p53 and p21WAF1 (**12**). The up-regulation of these proteins has been suggested to be an important mechanism by wich melatonin causes a cell cycle transition delay at the G_1-S interface. The accumulation of cells in G_1 induces them to enter in G_0, a quiescent state that leads the cells to undergo a higher differentiation; This suggest that the antiproliferative effects of melatonin on human breast cancer cells may be due in part to its ability to shift the balance from proliferation to differentiation. Moreover, melatonin inhibits hTERT, the human telomerase reverse transcriptase, which is the rate-limiting determinant of telomerase activity in breast cancer cells (**13**).

Melatonin also reduces the invasiveness of MCF-7 cells by decreasing their ability to attach to the basement membrane and by reducing their chemotactic response. Melatonin can also block the estradiol-induced invasion. Tumor cell motility and invasion are adhesion-dependent phenomena related to the presence of cell surface adhesion molecules. A down-regulation or a loss of expression of some of these surface adhesion molecules correlates with an increase in the invasiveness of tumor cells as well as with a poor cell differentiation and bad prognosis of the tumor progression. Melatonin increases the expression of β1-integrin and E-cadherin, two important molecules for cell-to-cell and cell-to-matrix interactions and then shift tumor cells to a lower invasive status by promoting their differentiation (**14**).

Finally melatonin exerts its modulatory effect in the tumor microenvironment by decreasing the secretion of cytokines by breast cancer cells, which regulates the differentiation and the aromatase expression and activity of fibroblasts surrounding malignant cells and also modulates aromatase activity and expression in endothelial cells. Melatonin may play a role in the paracrine interactions between malignant epithelial cells and proximal endothelial cells through a downregulatory action on VEGF expression in human breast cancer cells, which decreases the levels of VEGF around endothelial cells. Lower levels of VEGF could be important in reducing the number of estrogen-producing cells proximal to malignant cells and could also be important in reducing the tumor angiogenesis. VEGF secreted by breast cancer cells interacts with VEGF receptors in endothelial cells and stimulates downstream signaling molecules to promote the proliferation, growth, survival and migration of endothelial cells; therefore, inhibition of VEGF secretion by tumor cells, as well as VEGF regulated signaling in endothelial cells, could be important in reducing tumor angiogenesis and growth. Melatonin regulates tumor microenvironment by decreasing the secretion of VEGF by malignant epithelial cells and also regulates VEGF expression in human breast cancer cells (**15**).

In summary, the same molecule, melatonin, has properties to selectively neutralize the effects of estrogens on the breast and their local biosynthesis from androgens, one of the main goals of recent antitumor pharmacological therapeutic strategies. These actions collectively make melatonin an interesting anticancer drug in the prevention and treatment of estrogen-dependent tumors, since it has the advantage of acting at different levels of the estrogen-signaling pathways, both in tumor cells and the surrounding endothelial cells and fibroblasts.

1.3.- Melatonin and cancer: Clinical trials

As mentioned above, numerous experimental data from experiments performed *in vitro* (breast cancer cell-lines) as well as *in vivo* (animal models) have well established the oncostatic properties of melatonin. Since some investigations demonstrated an altered melatonin secretion in cancer patients, clinical trials have been conducted to evaluate the effects of melatonin in human neoplasms. After the pioneer clinical study of melatonin in untreatable advanced cancer patients performed by Lissoni et al (**16**), several studies have been published. It has been reported that melatonin protects hematopoietic precursors from the toxic effect of anticancer chemotherapy; thus, melatonin has been reported to attenuate damage to blood cells from both radio- and chemotherapy treatments. Moreover, melatonin may induce a decline in the frequency of chemotherapy induced asthenia, cardiotoxicity and neurotoxicity as well as an increase in the 1-year survival rate and the objective tumor regression in patients with metastatic solid tumors with poor clinical status. In metastatic non-small cell lung cancer patients treated with chemotherapy alone or chemotherapy with melatonin, both the overall tumor regression rate and the 5-year survival results were significantly higher in patients concomitantly treated with melatonin. Moreover, chemotherapy was better tolerated in patients who received the pineal hormone. The study suggests the possibility to improve the efficacy of chemotherapy in terms of both survival and quality of life by a concomitant administration of melatonin. A systematic review about the effect of melatonin in conjunction with chemotherapy, radiotherapy, supportive care, partial response, complete response, 1-year survival and chemotherapy-associated toxicities including data for 21 clinical trials, all of them with patients which dealt with solid tumors, concludes that melatonin may benefit cancer patients who are receiving chemotherapy. Patients who received melatonin showed substantial improvements in tumor remission, 1-year survival and ameliorating the side effects of chemotherapy. Yet another review summarising the data from 8 eligible randomized controlled trials (n=761) obtained similar conclusions (**17**).

1.4.- Chemotherapy

Chemotherapy consists in treatment with cancer–killing drugs, administered either intravenously or orally. Chemo drugs travel through the bloodstream to reach cancer cells in most parts of the body and work by targeting cells with a high rate of self-renewal which is a hallmark of cancer cells. Chemotherapy is recommended after surgery (adjuvant chemotherapy): surgery is performed to remove the tumor, and adjuvant therapy is given to kill any cancer cells that may have been left behind of spread but cannot be seen. Radiation, chemotherapy, targeted therapy and hormone therapy can all be used as adjuvant treatments. When treatments are administered before surgery instead of after, we speak of neoadjuvant therapy; the benefits of neoadjuvant chemo are that drugs may shrink the tumour so that it can be removed with less extensive surgery. Moreover, giving chemo before the tumour is removed, might help on subsequently monitoring the disease, in the case that the first cocktail of drugs do not diminish the tumour, other compounds might be considered. Finally, chemo is also used as main treatment for women whose tumour has spread outside the breast and underarm area. Combinations of drugs are often used to treat early breast cancer, while advanced disease is more often treated with single chemotherapy drugs. Chemo is usually given in cycles, with periods of treatment followed by resting periods to allow for recovery and minimize the side effects derived from the treatment **(Figure 1.4)**.

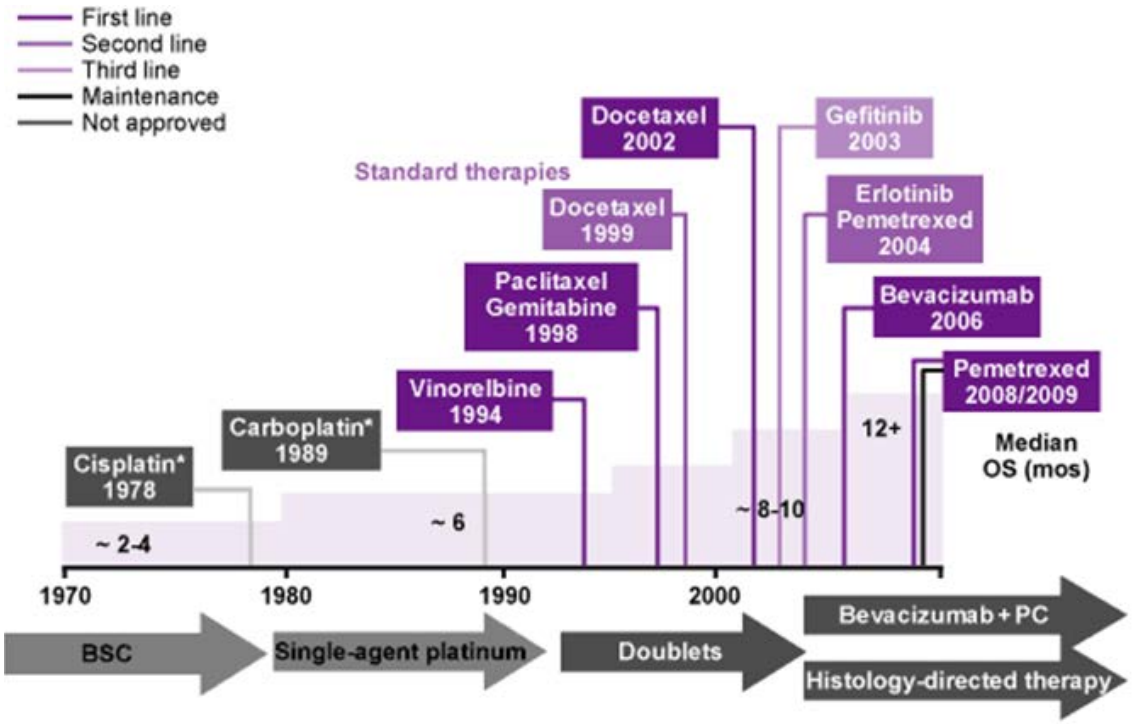

Figure 1.4: Most commonly used chemotherapy agents in breast cancer.

Anthracyclines are among the most effective treatments for various cancers. The most commonly used in breast cancer are epirubicin and doxorubicin. Epirubicin has been incorporated into

numerous combinations of chemotherapy in clinical trials. Pegylated doxorubicin liposomal doxorubicin is otherwise used in the treatment of breast and ovarian cancer, maintaining or improving the antineoplastic effects of doxorubicin but improving its toxicity profile.

Since microtubules play a role in the migration of chromosomes to opposite ends of mitotic cells during the anaphase, microtubule inhibitors (MIs) also known as microtubule-stabilizing agents (MSAs) are commonly used in the treatment of breast cancer; MI agents include microtubule depolymerizing agents (vinca alkaloids) and microtubule polymerizing agents (taxanes). Vinca alkaloids derive from the periwinkle plant *Catharantus roseus*. The first clinical trial demonstrating its efficacy in cancer was reported in 1963. Nowadays, these compounds are produced synthetically and include vinblastine, vincristine, vindesine and vinorelbine. The main mechanism of their cytotoxicity is mainly due to their interactions with tubulin and the subsequent disruption of microtubule function, particularly those microtubules comprising the mitotic spindle apparatus, directly causing metaphase arrest. These agents introduce a wedge at the interface of two tubulin molecules and thus interfere with tubulin assembly (**18**).

Taxanes are diterpenes obtained from *Taxus brevifolia*. The first taxane named paclitaxel was first isolated in 1967. However, they present difficulties in formulation because they are poorly soluble in water, for this reason, the first clinical trial including taxanes was not reported until 1987. The other taxane currently in use is docetaxel, obtained from European yew (*Taxus baccata*). Both paclitaxel and docetaxel act as a spindle poison, promoting microtubulin assembly and stabilising the polymers against depolymerization, leading to the inhibition of microtubule dynamics and consequently, to cell cycle arrest. The taxol-induced changes in tubulin conformation act against microtubule depolymerization in a precise directional way, binding a specific domain of tubulin (**Figure 1.5**) located in the internal surface of the microtubule (**19, 20**).

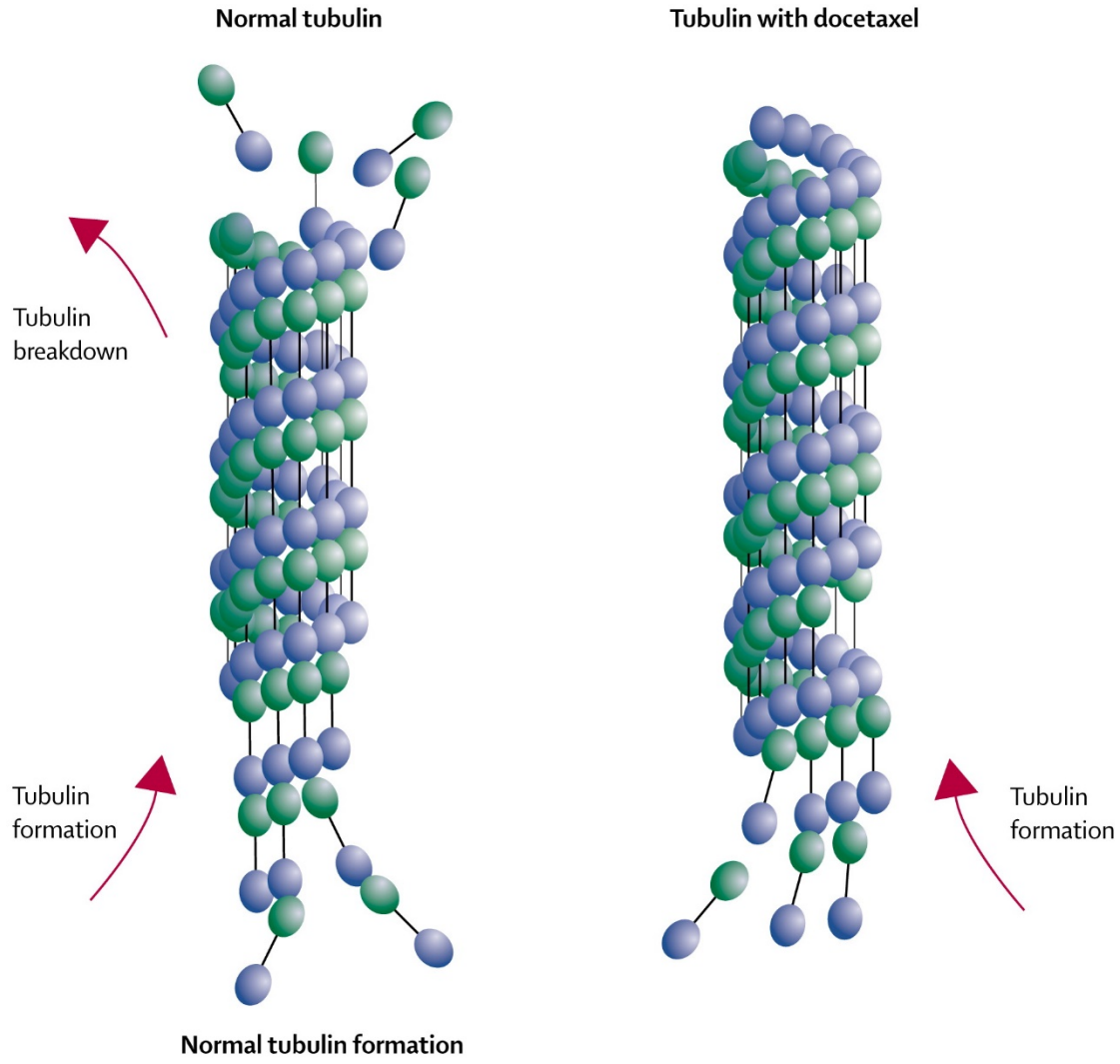

Figure 1.5: Docetaxel mechanism of action.

Other current approaches in the treatment of breast cancer include compounds developed against specific identified targets (molecular-targeted therapies) that contribute to the tumoral growth. Thus, trastuzumab is a monoclonal antibody used to treat metastatic breast cancer; it is effective against tumors that overexpress the HER2/neu protein. Despite the initial encouraging results of many therapeutic randomized trials that have been undertaken de novo and acquired resistence to trastuzumab can occur. Therefore, novel agents have recently been added to the list of drugs available to treat HER2-overexpressing breast cancers. One of them is lapatinib, a reversible inhibitor of EGFR and HER2/neu tyrosine kinases, approved in 2007 for patients with metastatic breast cancer resistant to trastuzumab. Another molecule is HKI-272, an irreversible pan-ERbB2 receptor tyrosine kinase inhibitor, which showed substantial clinical activity. HKI-272 targets a cysteine residue in the ATP binding pocket of the receptor resulting in the inhibition of downstream signal transduction events and cell cycle regulatory pathways. Unique within this

array of promising new agents are compounds that target heat shock protein (Hsp90) such as 17-AAG, has recently completed phase I testing. (**21**).

1.5.- Can melatonin enhance the beneficial and protect against the deleterious effects of chemotherapy?

As pointed above, melatonin has been shown to reduce the incidence of experimentally induced cancers and can significantly inhibit the growth of some hormone-dependent cancers. The antitumoral actions of melatonin have been observed in breast cancer, both in in vivo models, (in chemically induced rat mammary tumors), and in vitro studies (in human breast cancer cell lines). Also, there are many reports endorsing the beneficial use of melatonin during chemotherapy in clinical trials. Therefore, the ultimate goal of this review is to provide a survey on the current knowledge concerning the interplay of melatonin and chemotherapy agents at molecular levels. We searched in the PubMed database citations for 84 genes known to be commonly involved in the dysregulation of several normal processes during breast carcinogenesis, genes that are also in breast cancer cell lines. The list includes signal transduction genes and other commonly affected pathways such as angiogenesis, adhesion, proteolysis, cell cycle progression and control, and apoptosis (**table 1**); Research into carcinogenic mechanisms identified during the last decades many functional alterations due to somatic mutations, gene expression alterations and altered posttranslational protein modifications. Therefore, we performed the database search including as keywords: i) the name of each gene, ii) breast cancer, iii) melatonin and iv) chemotherapy.

For most of the genes reviewed, there are thousands of citations in the literature when the search includes the name of each gene and breast cancer as key words; when melatonin is included as selection criteria, the number of articles is drastically reduced; thus, 53 out of the 84 genes surveyed do not have a single report under these criteria. Only the estrogen receptor is near one hundred articles, when is searched together with melatonin and breast cancer; p53 and progesterone receptor have 15 and 12 reports respectively, and the rest of 28 genes go from 1 to 9 articles.

The number of reports is further reduced if chemotherapy is also included as a selection criteria; only 25 of the 84 genes are included in the list (**table 1**), meaning that out of 59 genes, there is not a single work published in which the gene, melatonin, breast cancer and chemotherapy are mentioned together (**table 1**).

Gene Name	Gene	Gene Br. cancer	Gene Melatonin	Gene Br. cancer Melatonin	Gene Br. cancer Melatonin CMT	Gene Cancer Melatonin CMT
ER	68885	31082	189	96	31	52
p53	78342	7698	80	15	7	12
p21	31647	2174	35	9	5	12
VEGF	57030	2737	64	12	3	11
PGR	33762	13582	73	12	3	5
TGFB1	22043	561	25	4	3	4
MYC	29932	1882	17	5	3	3
CDH1	23363	2200	13	5	3	3
IL6	96419	1106	162	3	2	10
GSTP1	35897	901	91	2	2	7
c-JUN	102575	2952	106	6	2	6
RARB	11545	651	76	9	2	5
CCND1	16332	2205	16	6	2	2
AR	22741	1690	64	3	1	11
AKT	54018	3992	92	6	1	10
Ki-67	20827	3129	18	5	1	5
ERK1	26166	1153	61	4	1	3
ERBB2	22268	14282	72	3	1	3
EGFR	35864	3550	7	3	1	3
IGFBP3	5003	432	6	2	1	2
EGF	29673	2215	24	6	1	2
Rb	19405	1017	16	1	1	1
IGF1	2700	160	15	1	1	1
CDK2	6054	491	5	1	1	1
p73	2089	113	1	1	1	1

Table 1. A search in PubMed data base was performed for 84 genes known to be altered in breast cancer. The keywords used were: a) "gene name", b) "gene name" AND "breast cancer", c) "gene name" AND "melatonin", d) "Gene name" AND "breast cancer" AND "melatonin", e) "Gene name" AND "breast cancer" AND "melatonin" AND "chemotherapy", f) "Gene name" AND "cancer" AND "melatonin" AND "chemotherapy". In the table, only the 25 genes that have at least one publication with the criteria "Gene name" AND "breast cancer" AND "melatonin" AND "chemotherapy" are shown. The genes that have no publications under these criteria of search are: Gelatinase A, PTGS2, Bad, Bcl2, BIRC5, Gelatinase B, CTNNB1, APC, ASC, ATM, ABCB1, ABCG2, BRCA1, TFF3, Cathepsin, μ-PA, SRC, PAI-1, Serpine1, JNK1, IGF1R, CDKN2A, ADAM23, PTEN, NOTCH1, THBS1, ID1, Keratin5, GATA3. ERK2, CCNE1, XBP1, NR3C1, BRCA2, MUC1, MLH1, Keratin19, NME1, TWIST1, FOXA1, RASFF1, HIC1, SFN, MGMT, CCND2, Cystatin, GRB7, Keratin8, GLI1, Keratin18, SFRP1, SNAI2, p57, CyclinA1, CDH13, CSF1, SLIT2, SLC39A6 and PRM2.

We conclude that, despite the fact that during decades many articles have reported experimental data from *in vitro* and *in vivo* experiments showing the oncostatic actions of melatonin, there are not many reports studying the effects of melatonin and chemotherapy agents in cancer treatment in general and therefore, in breast cancer in particular.

Similarly, little is known about the role of melatonin over the expression and functionality of the genes known to be altered in cancer (particularly in breast cancer). There is very little information available nowadays about gene expression profiles in cancer and particularly in breast cancer, and how the gene expression profile might be altered by treatment with different chemotherapy compounds and, remarkably, whether or not melatonin has protective effects when administered with chemotherapy agents. On the other hand, most of the information available about melatonin, cancer, chemotherapy and altered gene expression and function has been published in the last few years. This indicates that nowadays there is a growing field of research about this topic. Some of the most relevant findings recently reported concerning the modulatory role of melatonin in cancer at molecular level are the following: melatonin disruption by exposure to light at night drives intrinsic resistance to tamoxifen therapy in breast cancer. In this same line, it has also been described how a circadian disruption of nocturnal melatonin production contributes to a complete loss of tumor sensitivity to doxorubicin chemotherapy (**22**).

The resistance of cancer cells to chemotherapy treatments such as doxorubicin usually implicates an upregulation of the P-glycoprotein protein, responsible for the drug efflux from the cells; in this line, there is a report describing that melatonin treatment increase doxorubicin intracellular concentrations in cancer cells, suggesting that melatonin might inhibit the P-glycoprotein protein (**23**). Melatonin treatment results in Bim up-regulation and cyclooxygenase (COX-2) down-regulation, therefore enhancing tunicamycin-induced apoptosis in breast cancer cells. Modulation of COX-2, p300, Akt and Apaf-1 signalling by melatonin inhibits proliferation and induces apoptosis in breast cancer cells in vitro models. Melatonin drastically down-regulates MDM2 gene expression and inhibits MDM2 shuttling into the nucleus, since melatonin increases L11 and inhibits Akt-PI3K-dependent MDM2 phosphorylation. Melatonin induces an increase in both MDMX and p300 levels, decreasing simultaneously Sirt1, a specific inhibitor of p300 activity. Consequently, melatonin-treated cells display significantly higher values of both p53 and acetylated p53. Thus, a strong increase in p21 levels was observed in melatonin-treated cancer cells. It has been reported that melatonin sensitizes non-small-cell lung cancer cells harbouring an EGFR mutation to the tyrosine kinase inhibitor gefitinib. In combination with cisplatin, melatonin enhances cisplatin-induced cytotoxicity and apoptosis in lung cancer cells and cervical cancer derived HeLa cells. Consistently with these findings, cotreatment with melatonin and each of three different chemotherapy agents, namely 5-fluorouracil, cisplatin and doxorubicin resulted in melatonin enhanced chemotherapy-induced cytotoxicity and apoptosis in the rat pancreatic tumor cell line AR42J. In combination with the nucleoside analogue gemcitabine, recent results indicate that melatonin inhibits proliferation and invasion of pancreatic ductal adenocarcinoma cells through NF-κB inhibition.

Melatonin supports the effects of doxorubicin by activation of transient receptor potential vanilloid (TRPV1) and apoptosis inducing MCF-7 cell death. In a model of ovarian carcinoma, melatonin therapy promotes apoptosis along with the upregulation of p53, BAX and cleaved caspase-3, suggesting that melatonin induces apoptosis in ovarian cancer cells. In a gastric cancer cell line (AGS) p38, JNK and ERK are activated by melatonin treatment, which also significantly increases caspase-3 cleaved and Bax protein expression and decreased Bcl-2 protein expression; moreover, melatonin is able to strengthen the anti-tumour effects of cisplatin, with low systemic toxicity.

2.- Objectives

Melatonin is an oncostatic agent that reduces the growth and development of hormone-dependent tumors and enhances the efficacy of cancer chemotherapy. However, little is known about the effects that melatonin might have over the cellular and/or molecular changes induced by chemotherapy agents in breast cancer cells. Therefore, using ER+ MCF-7 breast cancer cells as a model, the objectives of this work were:

- To establish the changes in gene expression induced by docetaxel.
- To verify if melatonin is able to modulate those changes.
- To test the ability of melatonin to further stimulate the apoptosis triggered by docetaxel.
- To check if melatonin potentiates the antiproliferative effects of docetaxel used at subpharmacological doses.
- In summary, to check whether melatonin can be a beneficial adjuvant against breast cancer.

3.- Materials and Methods

3.1.- Cells and culture conditions

MCF-7 human breast cancer cells were purchased from the American Tissue Culture Collection (ATTC nº HTC-22™) (Rockville, MD, USA). They were maintained as monolayer cultures 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 (20 µg/ml) (Sigma-Aldrich, Madrid, Spain) at 37ºC in a humid atmosphere containing 5% CO_2.

Before each experiment cells were transferred to phenol red-free Dulbecco's medium containing 0.5% dextran-charcoal stripped FBS (scFBS) and maintained for 2 days. Then cells were washed twice using a balance salt solution (HBSS) and were detached from the culture plate by adding a solution containing 0.25% trypsin. After neutralization, cells were transferred to a 15 ml conical tube, centrifuged at 100 x g for 5 minutes and the pellet were resuspended in culture media, removing a sample for counting.

3.1.1.- Determination of cell number

The total number of cells and the viability were determined using a hemocytometer (Marienfeld, GmbH). Briefly, 100 µl of the cell suspension were pipetted into the counting chamber, underneath the coverslip, allowing capillary action to draw it inside. The full grid on a hemocytometer contains nine squares, each of which is 1 mm^2 (**figure 3.1**). The central counting area of the hemocytometer contains 9 large squares and each large square has 16 smaller squares. Using a fluorescence microscope (NIKON TMS), alive cells which were situated inside the four corners of the square and also the central square were counted. To calculate the number of cells in the initial cell suspension, we used the formula showed in **figure 3.1.**

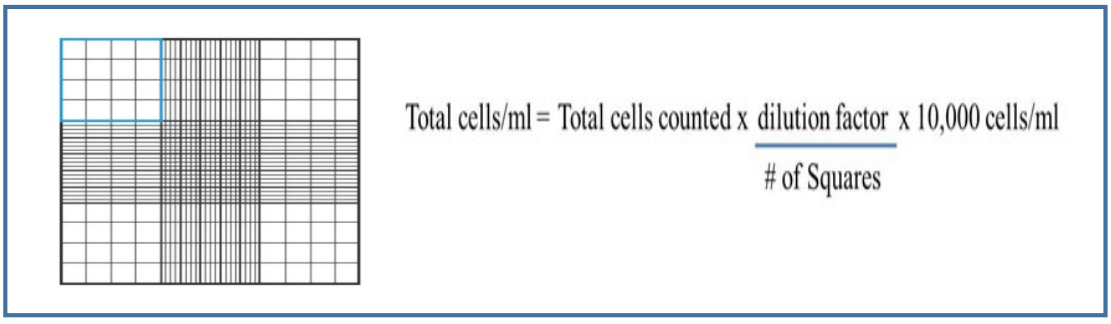

Figure 3.1. Hemocytometer gridlines. Hemocytometer diagram indicating one of the sets of 16 squares that should be used for counting.

3.2.-Reagents and treatments

Docetaxel (Sigma-Aldrich) is a taxane anti-cancer agent that binds and stabilizes the β−tubulin subunit of microtubules, preventing depolymerization of the mitotic spindle. Docetaxel were diluted in ethanol at a final concentration of 0.1 M (stock) and stored refrigerated. When this taxane were added to the culture media, the final ethanol concentration were lower than 0.001%. Melatonin stock (Sigma-Aldrich) were diluted also in ethanol at a final concentration of 100 mM and stored aliquoted at -20ºC. Melatonin were used at a final concentration of 1 nM in the culture media, which is a dose considered equivalent to the physiologic concentration 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 docetaxel, 1 nM melatonin, docetaxel plus melatonin or vehicle (ethanol at a final concentration lower than 0.001%). Cells were incubated for 6 hours at 37º 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 were 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 -80ºC. 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.

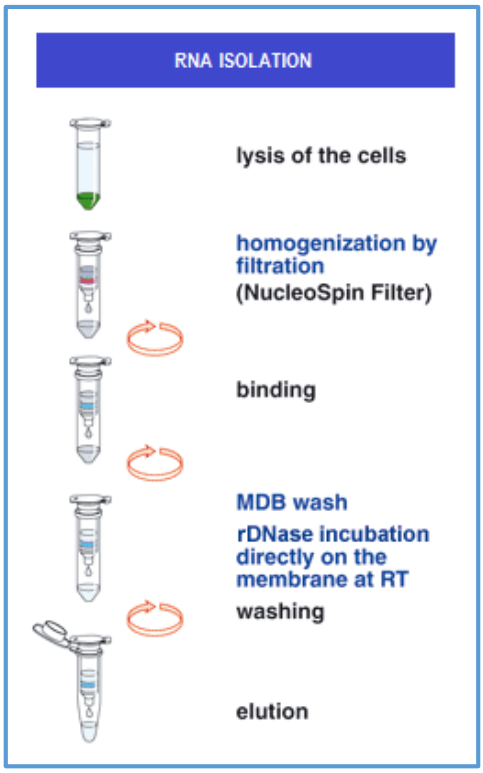

Figure 3.2: Steps of RNA isolation method using RNA NucleoSpin II commercial kit.

For cDNA synthesis, 0.5 µg of total RNA was used as template using the RT^2 First Stand Kit (Qiagen, USA), following the manufacturer´s instructions (**table 3.1**). First, the genomic DNA was eliminated by incubating the samples 5 min at 42 ºC. After mixing with the reverse-transcription mix, the samples were incubated for exactly 15 min at 42 ºC in a final volume of 20 µl. The reaction was stopped by incubating at 95 ºC 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.- RT^2 Profiler PCR array

Pathway-focused gene expression profiling was performed using a 96-well human breast cancer PCR array (RT^2 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 enzymes 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 phosphoribosyltransferase1 (HPRT1) and ribosomal protein, large, P0 (HPLP0). 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 3.3**). The PCR array performs gene expression analysis with real-time PCR sensitivity and the multi-gene profiling capability of a microarray. 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 (**figure 3.4**). Amplifications were initiated by 1 cycle at 95ºC for 10 min and then performed for 45 cycles for quantitative analysis using the following temperature profile: 95ºC for 30 sec (denaturation); 60ºC for 60 sec (annealing/extension), setting the instrument to detect and report fluorescence at each cycle during the 60ºC annealing/extension step. Melting curves were performed by using dissociation curve to verify that only a single product with no primer-dimers was amplified.

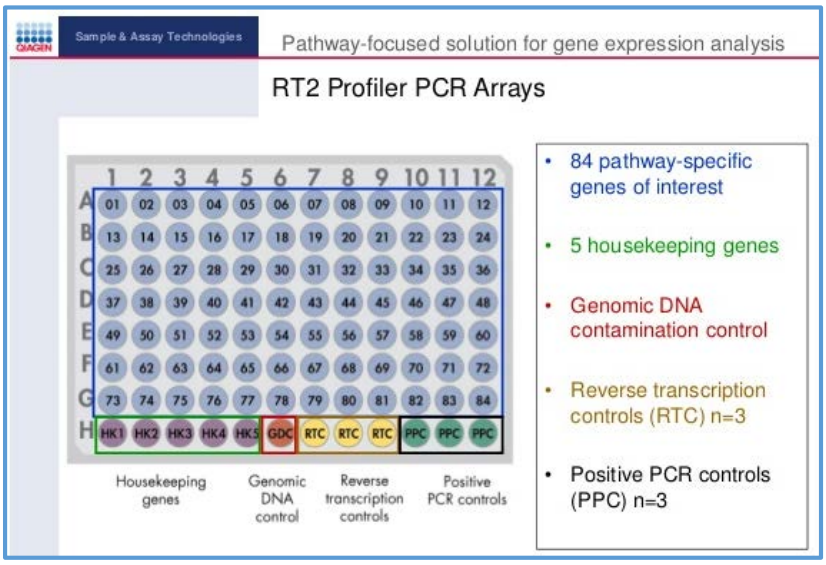

Figure 3.3: Scheme of a RT^2 Profiler™ PCR Array (Qiagen).

3.5.1.- qPCR Data analysis

The $\Delta\Delta C_T$ method is recommended for data analysis. In separate reactions, the C_T value 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 C_T 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 ($\Delta\Delta$CT) 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 C_T 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) – $_{CT}$ (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 $2^{(-\Delta\Delta CT)}$.

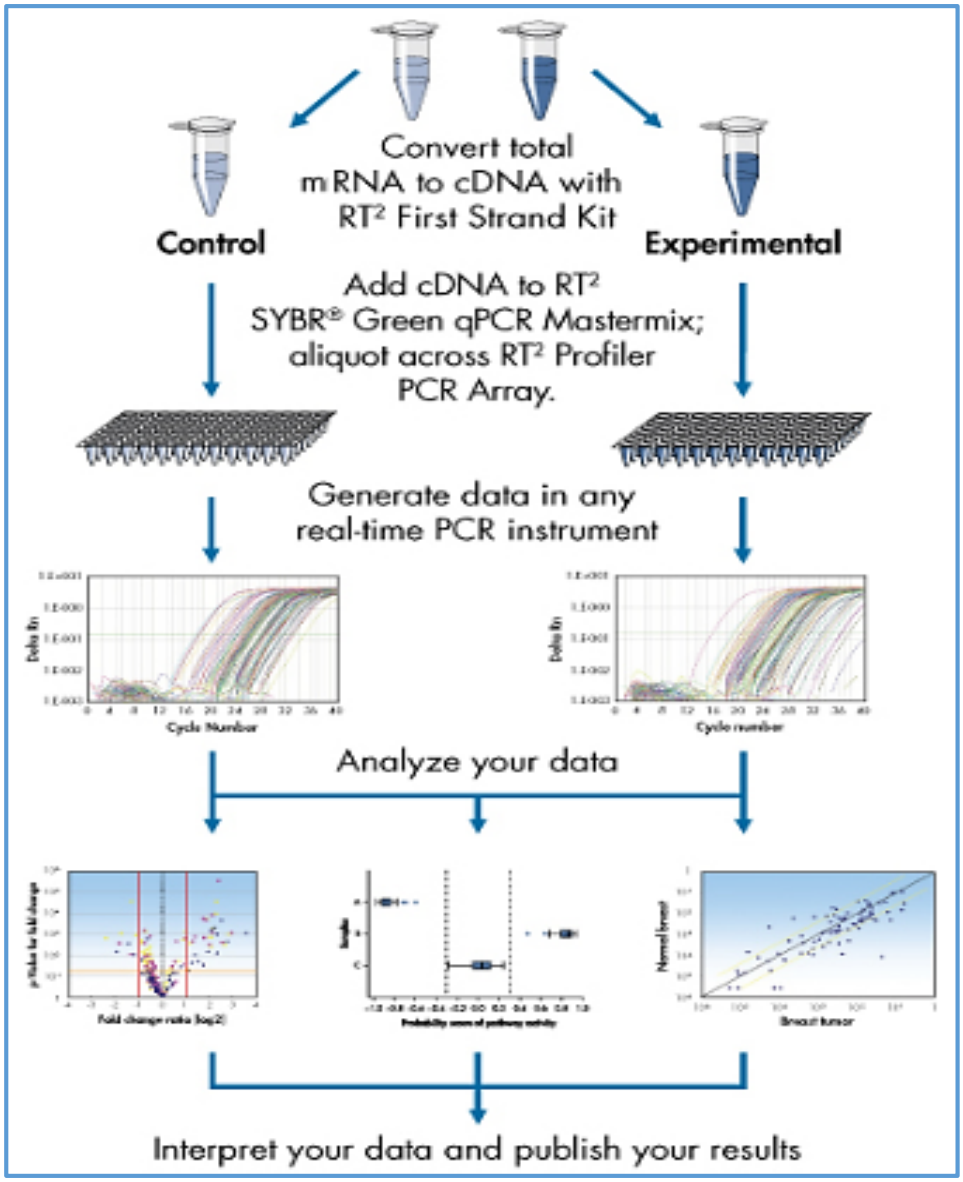

Figure 3.4: Procedure for the quantitative determination of gene expression of 84 genes related to breast cancer, using RT² Profiler™ PCR Array plates.

3.6.- Measurement of BAD, BAX, BCL-2, CDKN1A, GATA3, MUC1, MYC, p53, CDH13 mRNA gene expression

Specific analysis of the *BAD*, *BAX*, *BCL-2*, *CDKN1A (p21)*, *GATA3*, *MUC1*, *MYC*, *p53* and CDH13 mRNA gene expression in MCF-7 cells was carried out by qRT-PCR. Primers used for amplification of *BAD*, *BAX*, *BCL-2*, *CDKN1A*, *GATA3*, *MUC1*, *MYC*, p53 (Sigma Genosys Ltd, Cambridge, UK) and *CDH13* (Qiagen, USA) were designed so that the coding sequence between the two PCR primer sites is interrupted by at least one intron in the gene. For the primer design, the following programs were used: *SequenceAnalysis 1.6; DNAMAN (Lynnon Corporation); SequenceManipulationSuite*. In order to optimize primer concentration, a matrix of six concentrations of each pair (50 nM to 400 nM) were performed.

qRT-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 sample was calculated by the $2^{-\Delta\Delta Ct}$ method.

The oligonucleotides used are shown in **table 3.2**, except for *CDH13* gene which was distributed by Qiagen:

GENE	PRIMER SEQUENCE
β-actin forward	*5´-TCCTGCGAGTGCTGTCAGAG-3´*
β-actin reverse	*5´-TCACCGCCCTACACATCAAAC-3´*
p53 forward	*5´-CAGCCAAGTCTGTGACTTGCACGTAC-3´*
p53 reverse	*5´-CTATGTCGAAAAGTGTTTCTGTCATC-3´*
CDKN1A forward	*5´-CAGCATGACAGATTTCTACC-3´*
CDKN1A reverse	*5´-CAGGGTATGTACATGAGGAG-3´*
BAX forward	*5´-AACTGGACAGTAACATGGAG-3´*
BAX reverse	*5´-TTGCTGGCAAAGTAGAAAAG-3´*
BAD forward	*5´-ATCATGGAGGCGCTG-3´*
BAD reverse	*5´-CTTAAAGGAGTCCACAAACTC-3´*
BCL2 forward	*5´-CCTTTGGAATGGAAGCTTAG-3´*
BCL2 reverse	*5´-GAGGGAATGTTTTCTCCTTG-3´*
MUC1 forward	*5´-GCAAGAGCACTCCATTCTCAATT-3´*
MUC1 reverse	*5´-TGGCATCAGTCTTGGTGCTATG-3´*
GATA3 forward	*5´-CGGTCCAGCACAGGCAGGGAGT-3´*
GATA3 reverse	*5´-GAGCCCACAGGCATTGCAGACA-3´*
MYC forward	*5´-TGAGGAGGAACAAGAAGATG-3´*
MYC reverse	*5´-ATCCAGACTCTGACCTTTTG-3´*

Table 3.2: Primers used for amplification 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 37ºC for 24 hours to allow for cell attachment. Melatonin pretreated 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 docetaxel at different concentrations (1 µM, 100 nM, 10 nM, 1 nM, 0,1 nM, 0,01 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 3 or 6 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.- Determination of apoptosis

In most normal, viable eukaryotic cells, the negatively charged phospholipid phosphatidylserine (PS) is located in the cytosolic leaflet of the plasma membrane lipid bilayer. PS redistribution from the inner to the outer leaflet is an early and widespread event during apoptosis. Annexin V is a 35 kDa phospholipid-binding protein and a major cell membrane component of macrophages and other phagocytic cell types. Annexin V has a high affinity to PS in the presence of physiological concentrations of calcium (Ca^{2+}).

Induction of apoptosis was determined using an Annexin V-FITC apoptosis detection kit (Miltenyi Biotec GmbH, Germany), according to the manufacturer´s instructions. Cells were initially cultured for 24 hours in DMEM supplemented with 0.5% dextran-charcoal stripped FBS (csFBS) before being seeded into 6-well plates in DMEM supplemented with 10% FBS and incubated at 37ºC for 24 hours to allow for cell attachment. Then, media were replaced by fresh ones with 10% FBS and containing 10 nM docetaxel, 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%). After 24 hours incubation, cells were harvested, washed twice with phosphate-buffered saline (PBS), and centrifuged at 300 x g for 10 minutes; then, the supernatant was discarded, and the pellet was resuspended in 100 µl of propidium iodide (PI). The cells were

immediately analyzed after incubation with the probes by flow cytometry (Becton Dickinson FACS CANTO II analyzer). Ten thousand events were analyzed using the FL-1 (green; annexin V-FITC) and FL-3 (red; PI) detectors. Each sample was tested three times in independent experiments. Under all conditions tested, the percentages of annexin$^-$/PI$^-$ (alive cells), annexin$^+$/PI$^-$ (early apoptotic) and annexin$^+$/PI$^+$ cells (late apoptotic) were mainly compared.

3.9.- Statistic analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical differences between groups were processed by One Way Analysis of Variance (ANOVA) followed by the Student-Newman-Keuls test.

Results were considered as statistically significant at $p < 0.05$. Data were expressed as percentage of the control group (mean ± SEM). In the figures, letters a, b and c represent the significance level over control groups; **(a)** $p < 0.05$; **(b)** $p < 0.01$; **(c)** $p < 0.001$. Letters d and e represent the significance level versus docetaxel 1 nM groups; **(d)** $p < 0.01$; **(e)** $p < 0.001$ and f represents the significance level versus the 0.1 nM docetaxel groups **(f)** $p < 0.001$.

4.- Results

4.1.- Study of gene expression

To study the effects of docetaxel (1 µM) and melatonin (1 nM) on the dysregulation of signal transduction in the breast cancer cell line MCF-7 we used the human breast cancer RT² Profiler PCR Array, which contains 84 key genes involved in different pathways commonly altered in breast carcinogenesis. As expected, there were many genes whose expression was modified when MCF-7 cells were treated with docetaxel. Also, when cells were treated with a combination of docetaxel and melatonin at physiological concentrations (1 nM), we observed significant changes in the expression profile of some other genes. **Table 4.1** shows those genes that changed their expression (up-regulated or down-regulated) at least ±50% compared to control cells.

DOCETAXEL			DOCETAXEL + MELATONIN			
UPREGULATED	DOWNREGULATED		UPREGULATED		DOWNREGULATED	
BIRC5	ABCB1	GSTP1	ATM	MAPK8	ABCB1	GATA3
EGFR	ADAM23	HIC1	CCND1	MUC1	ADAM23	GLI1
KRT18	AR	IGF-1	CCND2	MYC	BRCA2	IGF1
KRT19	BAD	IGFBP3	CCNE1	NOTCH1	CDH13	IL6
NOTCH1	BCL2	KRT5	CDK2	PGR	CDKN1A	KRT5
PGR	CDH13	KRT8	CTSD	PTEN	CDKN2A	MMP2
PLAU	EGF	MMP2	EGFR	RASSF1	CST6	PTGS2
PTEN	GATA3	SLIT2	ESR2	SERPINE1	CATENIN	SFRP1
SERPINE1	GRB7	SRC	HIC1	SNAI2	FOXA1	P73
TFF3	GSTP1		IGFR1	TFF3		
VEGFA			JUN	P53		
XBP1			KRT18	VEGFA		

Table 4.1: Up-regulated (green) and down-regulated (red) genes. MCF-7 cells were treated with 1 µM docetaxel and/or 1 nM melatonin for 6 hours. The table shows those genes whose expression level was modified ± 0.5compared to control, calculated by the $2^{-\Delta\Delta Ct}$ method.

It is important to note that these PCR array contains only one well for each of the genes analyzed, so the results are indicative of what may be happening with each of the treatments but there is only one data for each of the conditions tested. Thus, these results must be confirmed by specific qPCR.

For this purpose, nine of the most representative genes showed in table 4.1 were selected to be analysed by specific qPCRs after incubating cells with docetaxel or docetaxel + melatonin for 6 h. The results are showed in **figure 4.1.**

Docetaxel treatment significantly decreased the expression levels of the tumor supressor genes p53 and p21. Interistingly, combination of this chemotherapeutic agent with melatonin counteracted the docetaxel effect, increasing mRNA expression levels.

Cdh-13 is a gene involved in cell adhesion, which is important for tumor spread and metastasis. Docetaxel itself decreased its expression, but when administered with melatonin had the opposite effect.

Regarding the genes involved in apoptosis, docetaxel treatment induced and increase on the gene expression of two pro-apoptotic genes (Bax, Bad), decreasing the expression of the anti-apoptotic gene Bcl-2. Melatonin significantly potentiated the effects of docetaxel on the mRNA gene expression of Bax and Bad, and also decreased even more the expression of Bcl-2.

Finally, docetaxel treatment resulted in an increased gene expression of the transcription factors Muc1, Gata3 and Myc. However, when cells were treated with docetaxel and melatonin, this hormone was able to counteract the stimulatory effects of the taxane.

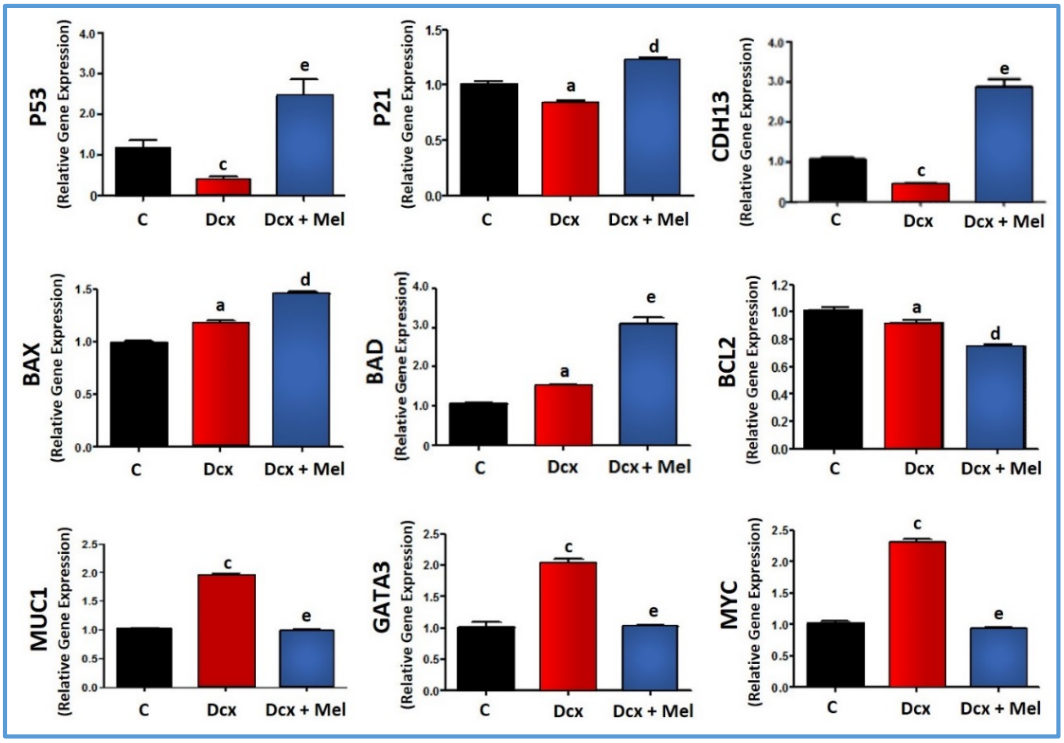

Figure 4.1: Effects of docetaxel and melatonin on gene expression p53, p21, Cdh13, Bax, Bad, Bcl-2, Muc1, Gata3 and Myc. Cells were treated with 1 μM docetaxel and/or 1 nM melatonin for 6 h and total mRNA was isolated and reverse transcribed. cDNA was subjected to qRT-PCR using specific primers, and β-actin as a housekeeping gene. Data are expressed as a relative gene expression compared to control cells. **(a)** $p < 0.05$, **(c)** $p < 0.001$ versus control; **(d)** $p < 0.01$, **(e)** $p < 0.001$ versus docetaxel 1 nM.

4.2.- Measurement of cell proliferation

The next objective of this work was to study if melatonin potentiates the antiproliferative effects of docetaxel on MCF-7 cells. When cells were treated with the docetaxel dose used for the gene expression assays (1 µM), we found that the proliferation rate was reduced by almost 80% within 3 days of culture. Thus, this dose was too high and did not allow us to study if melatonin was able to potentiate docetaxel effects.

For this reason, we first wanted to determine the effects of different docetaxel doses on MCF-7 cell proliferation. As expected, docetaxel inhibited cell proliferation in a dose dependent manner. As we can see on the **figure 4.2.**, doses of 10 nM or above induced a significant decrease on cell proliferation at 6 days of incubation. However, when cells were treated with docetaxel 0.1 nM or 0.01 nM, the antiproliferative effect of this chemotherapeutic agent was significantly reduced, being the cell proliferation rate similar to that of the control group. Based on these results, we selected subpharmacological doses of docetaxel (1 and 0.1 nM) to test if melatonin could modify the antiproliferative effects of this taxane.

The results of the growing cells treated with docetaxel and melatonin are shown in **figure 4.3**. Interstingly, melatonin at physiological concentrations (1 nM) potentiates the docetaxel-induced growth inhibition, both at 1 nM and 0.1 nM dose. Moreover, pretreatment of breast cancer cells with melatonin for 24 h prior docetaxel led to a significantly higher decrease of cell proliferation compared to non pretreated cells.

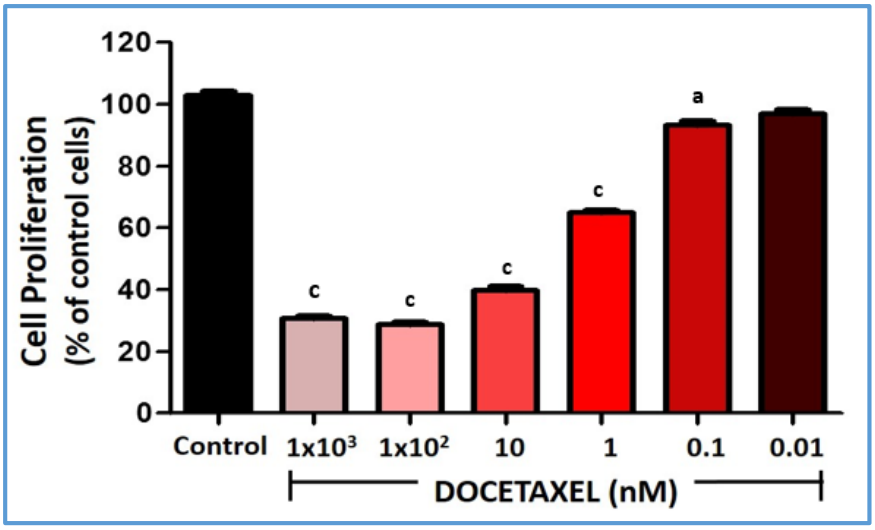

Figure 4.2: Effects of docetaxel on MCF-7 cell proliferation. MCF-7 cells were cultured into 96-multiwel plates in DMEM supplemented with 10% FBS and different docetaxel doses. After 3 days cell proliferation was measured by the MTT method. Data are expressed as the percentage of the control group (mean±SEM). **(a)** $p < 0.05$, **(c)** $p < 0.001$ versus control.

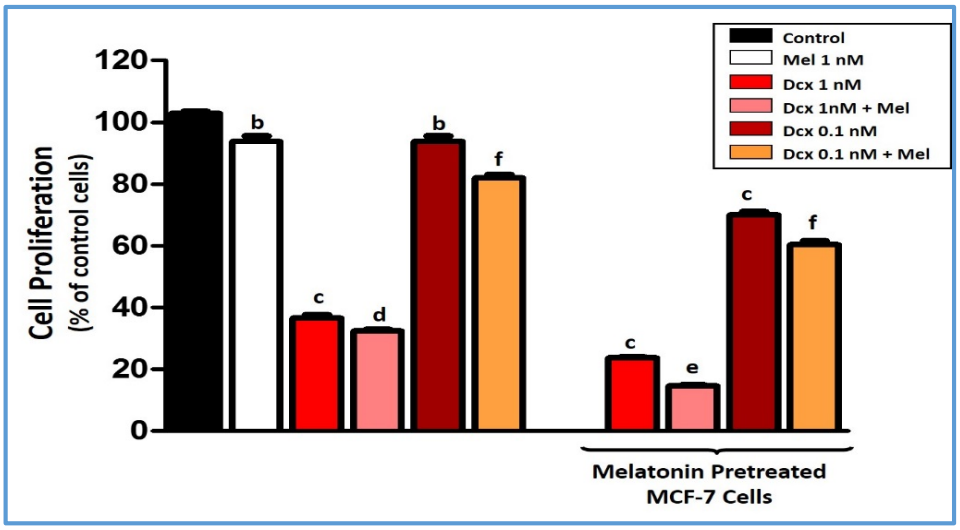

Figure 4.3: Potentiation of docetaxel-induced growth inhibition by melatonin. Effects of docetaxel (DCX) (1 nM, 0.1 nM) and/or 1 nM melatonin (Mel) on MCF-7 cells proliferation after 6 days of culture. Data are expressed as percentage of the control group (mean ± SEM). **(a)** $p < 0.05$; **(b)** $p < 0.01$; **(c)** $p < 0.001$ versus control; **(d)** $p < 0.01$; **(e)** $p < 0.001$ versus 1 nM docetaxel groups **(f)** $p < 0.001$ versus 0.1 nM docetaxel.

4.3.- Determination of apoptosis

To examine whether the antiproliferative effects of docetaxel and melatonin was related to induction of apoptotic cell death, we used an Annexin V-FITC apoptosis detection kit which allow to differentiate allows between viable cells, early apoptotic cells, late apoptotic cells and necrotic cells.

For this purpose, cells were treated during 24 h with a subpharmacological dose of docetaxel (0.1 nM), alone or in combination with 1 nM melatonin. The effect of melatonin pretreatment was also tested, being cells pretreated during 24 h before adding docetaxel to the culture media. Results are shown in **figure 4.4**. Actually, when cells were treated with melatonin alone, we observed a slightly decreased in the percentage of alive cells compared to control cells. Similarly, docetaxel induced a significant decrease of the number of cells alive, increasing the percentage of cells in early apoptosis. Interstingly, when cells were treated with docetaxel and melatonin, this result was potentiated, thus melatonin improved the apoptotic effect of docetaxel. Once again, melatonin pretreatment was even more effective than docetaxel alone in reducing the percentage of alive cells.

These findings suggested that melatonin potentiates docetaxel-stimulated cells, thereby allowing them to enter mitochondrial apoptosis faster.

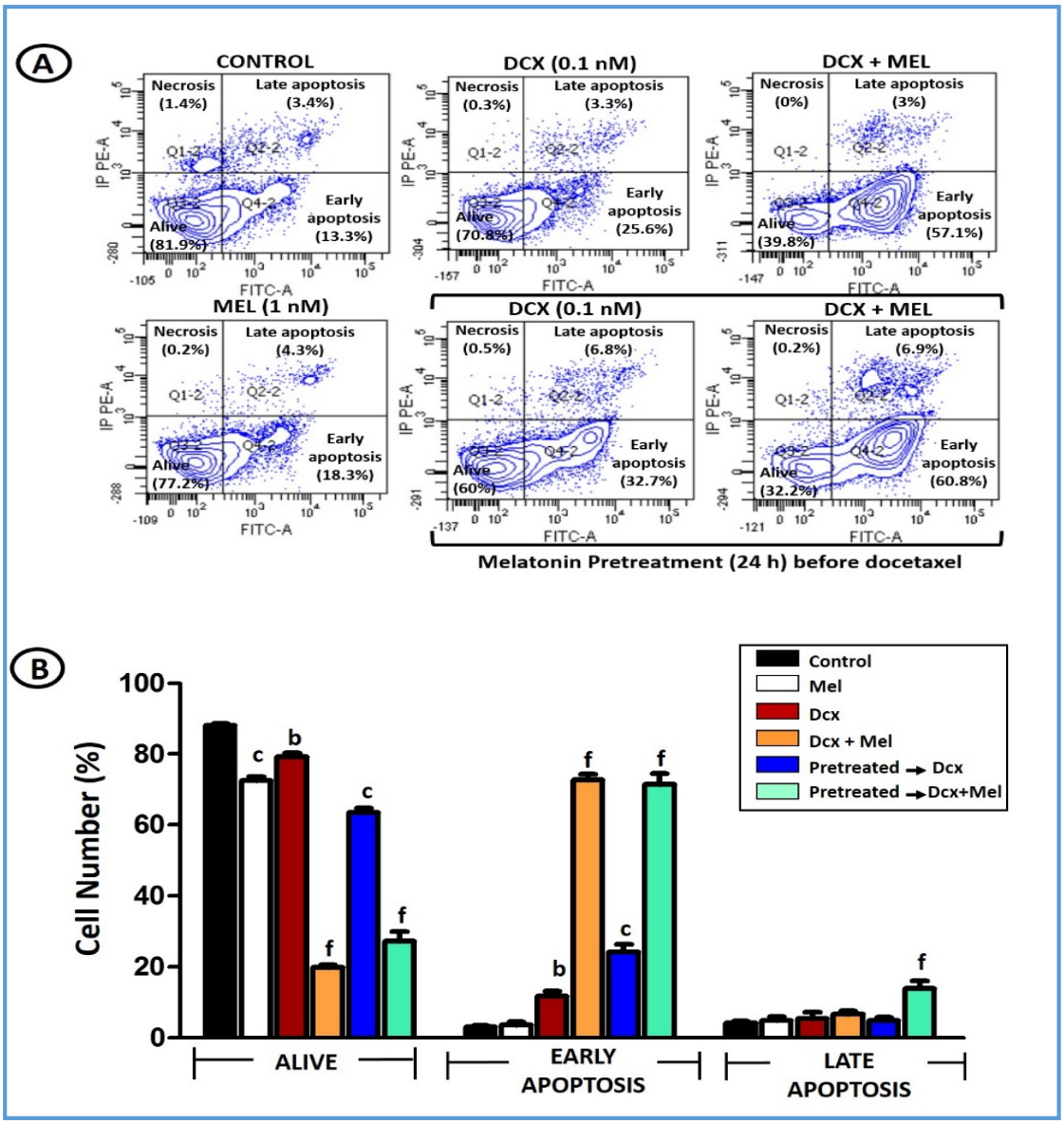

Figure 4.4: Potentiating effect of melatonin on docetaxel-induced apoptosis in MCF-7 cells. **A)** Representative dot-plots showing viable cells (Annexin$^-$/IP$^-$), early apoptotic (Annexin$^+$/IP$^-$) and late apoptotic (Annexin$^+$/IP$^+$) after 24 hours of treatment with docetaxel (0.1 nM) and/or melatonin (1 nM). **B)** Histograms showing percentages of each population. Values are presented as means ± SEM. **(b)** $p < 0.01$; **(c)** $p < 0.001$ versus control; **(f)** $p < 0.001$ versus 0.1 nM docetaxel.

5.- Discussion

Breast cancer is the most common cancer among women worldwide. Nowadays, 1 in 8 women will develop breast cancer during her lifetime. It is the second leading cause of deaths by cancer among women only exceeded by lung cancer. This represents 12% of all new cancer cases and 25% of all cancers in women.

Breast cancer is hormone related; most of the mammary tumors, at least two out of every three cases of breast cancer are hormone receptor positive. The tumor is called estrogen-receptor-positive (ER+) if it has receptors for estradiol. It will be classified as progesterone-receptor-positive (PR+) if it has receptors for progesterone. Estrogen receptor status is also a prognostic factor. (ER+) tumors tend to be linked to better survival than tumors with few or no estrogen receptors.

The estrogen receptor status helps to choose the treatment for breast cancer. When the mammary tumor is (ER+), the pharmacological protocol pretends to neutralize the effects of the estrogens on the breast. Hormonal therapy includes compounds that block estrogen from supporting the growth, proliferation and function of breast cells. These medications are called selective estrogen receptor modulators (SERMs), substances that bind and block the estrogen receptor preventing the estrogen binding, therefore acting as estrogen antagonists. The most commonly used in clinica are tamoxifen and fulvestrant. They act at the transcriptional level, impairing the transcription of many genes that are activated after the binding estradiol-ER receptor. At the moment, tamoxifen is administered to premenopausal, whereas fulvestrant is given to postmenopausal women. Fulvestrant has the advantage over other SERMs, since it does not have agonistic effects in other tissues (as tamoxifen, for example, does) known to have several secondary effects including an increase in the risk of developing endometrium cancer.

Another possibility is to use selective estrogen enzyme modulators (SEEMs). It is well established that mammary tumors express all the enzymatic machinery for the local synthesis of estrogens. The high incidence of (ER+) breast cancer in postmenopausal women suggest an important role of extragonadal synthesis of estradiol in breast carcinoma tissues. For this reason, there is a great interest in developing drugs capable to interfere with the local synthesis of steroid hormones by inhibiting the enzymes necessary to convert androgenic precursors into estrogens. As examples of such modulators, we can cite formestane (Lentaron) and anastrozole (Arimidex) that are currently in clinical use as second-line treatments for advanced breast cancer. They act as inhibitors of the enzyme complex aromatase; the aromatase activity in breast cancer tissues is higher than in nonmalignant breast tissues, fact that leads to the hypothesis that an increased

production of estrogens within breast tumors may exert a biological effect potentiating tumor growth specially in postmenopausal women.

Melatonin is the main product of the pineal gland; it has been well documented that this indolamine can inhibit the growth of different kinds of neoplasia, especially breast cancer, by interacting with estrogen-responsive pathways, therefore behaving as an antiestrogenic hormone. Many experiments have been conducted in tumor cell lines in *vitro* and animal models *in vivo,* leading to the conclusions that indeed, melatonin at physiological concentrations, is capable of downregulate the expression and activity of the enzymes necessary for the synthesis of estrogens from androgenic precursors, therefore behaving as a SEEM. Thus, in MCF-7 cells, melatonin reduces both the activity and expression of the aromatase;

Also, melatonin decreases the expression of ERα and impairs the estrogen-dependent transcriptional activation through destabilization of the estradiol-ER complex, thus behaving as a SERM. Therefore, the same molecule comprise SERM and SEEM properties. It seems that melatonin exerts its antiproliferative effects on breast tumors by modulating the expression of many growth factors, proto-oncogenes, and increasing the expression of other genes such as p53 and p21WAF1.

Moreover, melatonin inhibits hTERT, the human telomerase reverse transcriptase. This indolamine also exerts regulatory effects in the tumor microenvironment by decreasing the secretion of citokines by breast cancer cells, wich in turn regulate the differentiation, aromatase expression and activity of the fibroblasts surrounding malignant cells. Moreover, melatonin plays a role in the paracrine interactions between malignant epithelial cells and proximal endothelial cells, by decreasing the levels of the vascular endothelial growth factor (VEGF) that otherwise promotes proliferation, growth, survival and migration of endothelial cells. Thus, melatonin seems to inhibit both the tumor growth, the capability of malignant cells to migrate, invade and metastasize.

All this promising properties of melatonin as an oncostatic agent in ER+ tumors stimulated several investigators dealing with patients to evaluate the effects of the hormone in clinical trials. The conclusions are that patients receiving chemotherapy showed improvements in tumor remission, survival and ameliorating the side effects of chemotherapy.

There are many genes known to be altered during carcinogenesis, including genes involved in signaling transduction, angiogenesis, adhesion, control of cell cycle progression and apoptosis. When we search on the PubMed database for citations for 84 genes known to be altered in breast cancer, including as keywords the name of the gene, breast cancer, melatonin and chemotherapy, we found that only 25 of the 84 genes appear under these criteria of search. There is very little information available about the effects of melatonin and chemotherapy when used at the same

time on the regulation of those genes. Therefore, one of the first goals of this work was to perform a screen of the expression profiles in the breast cancer cell-line MCF-7 when cells were co-treated with melatonin and docetaxel, a chemotherapy agent commonly used in breast cancer treatment.

Docetaxel is a spindle poison that belongs to the family of taxanes, compounds that promote microtubulin assembly and stabilize the polymers against depolymerization, leading to the inhibition of microtubule dynamics, and consequently, to cell cycle arrest.

MCF7 cells were treated with pharmacological doses of docetaxel and physiological doses of melatonin for 6 hours, and total cellular RNA was extracted followed by cDNA synthesis. Next, a pathway-focused gene expression profiling was performed using a human breast cancer PCR array, including 84 genes which expression is known to be altered in breast cancer, and that also includes several positive and negative controls of transcription, as well as housekeeping controls to refer to, since their expression is not altered by the treatments.

12 out of the 84 genes were up-regulated when MCF-7 cells were treated with docetaxel and 19 were down-regulated (at least 50% up or downregulation compared to control). When cells were treated simultaneously with melatonin and docetaxel, the list of upregulated genes is of 24 and 18 were downregulated. As the PCR array contains only one well for each of the genes analyzed, the results give only a clue of what might be happening with the expression, but it is necessary to confirm the results by specific qPCRs.

During the realization of the following objective of this work, we obtained data to confirm the results for 9 of the genes included in the list of genes up or downregulated at least a 50% accordingly to the profiling expression experiment. Docetaxel treatment significantly decreased the expression levels of the tumor suppressor genes p53 and p21 and the adhesion molecule cadherin-13, which is downregulated in tumor spread and metastasis. Interestingly, melatonin counteracts the effects of docetaxel, increasing mRNA expression levels of p53, p21 and CDH13. The results point to a positive role of melatonin. High levels of CDH13 correlate with a lower ability of the cells to lose adhesion, migrate and invade other tissues (**24**). The tumor suppressor gene p53 is a central factor in the maintenance of genome stability. P53 induces a transcriptional program that results in cell cycle arrest, DNA repair, senescence and apoptosis; moreover, it has been recently demonstrated that p53 is a negative regulator of aromatase in the breast (**25**). In summary, melatonin seems to have a positive effect on the expression of three important genes involved in cell cycle control and adhesion.

Concerning to genes involved in apoptosis, we analyzed the expression of the pro-apoptotic genes Bax and Bad, and the anti-apoptotic gene Bcl-2. The BAX gene encodes a protein named Bcl-2-associated X protein. It is a pro-apoptotic member of the Bcl-2 protein family. The expression of this gene is up-regulated by the tumor suppressor P53 and BAX has been shown to be involved

in P53-mediated apoptosis. BAD gene encodes a protein named Bcl-2-associated death promoter. It is a pro-apoptotic member of the BCL-2 gene family involved in initiating apoptosis. When this protein is activated, it is able to form a heterodimer with anti-apoptotic proteins (like BCL-2) inactivating them and prevent them from stopping apoptosis. Thus, BAD allows Bax-triggered apoptosis (**26**). BCL-2 encodes a protein named B-cell lymphoma 2. It is considered an important anti-apoptotic protein and it is classified as an oncogene (**27**).

Treatment with docetaxel increased the expression of Bax and Bad (the pro-apoptotic genes) and reduced the levels of Bcl-2 (the anti-apoptotic gene). These results make sense since docetaxel prevents actively dividing cells to successfully accomplish mitosis. Importantly, melatonin potentiated the stimulatory effects of docetaxel on the expression of Bax and Bad, and further inhibited the anti-apoptotic expression of Bcl-2. All these results together suggest that cells are forced to enter into apoptosis by docetaxel, and melatonin potentiates the apoptotic effect of this chemotherapy agent.

The last three genes studied were MUC1, GATA3 and Myc. MUC1 encodes a glycoprotein (mucin1) of cell surface association. Overexpression of MUC1 is often associated with different types of cancer, where MUC1 promotes cancer cell invasion through beta-catenin, resulting in the initiation of epithelial-mesenchymal transition, which promotes the formation of metastases. GATA3 gene encodes a transcription factor named trans-acting T-cell-specific. It regulates luminal epithelial cell differentiation in the mammary gland. In breast cancer, GATA3 participates in estrogen receptor α signaling pathways (**28**).

MYC gene encodes Myc protein. It is a transcription factor, a phosphoprotein associated with cell cycle progression. It promotes cell proliferation (downregulates p21). MYC is a very strong proto-oncogene and it is commonly upregulated in many types of cancers. We found that docetaxel induces the expression of these 3 genes, whereas melatonin counteracts the stimulatory effects of this taxane. Again, melatonin seems to exert a protective effect, since the inhibition of the expression of MUC1, GATA3 and the proto-oncogen Myc might well correlate with lower levels of expression of factors involved in cellular growth and a less aggressive invasion phenotype (**29**).

The global interpretation of the results of the 9 genes specifically tested, is that melatonin influences gene expression altered by docetaxel in MCF-7 cells, in a way that fits well with its antiestrogenic and antitumoral actions.

Next we decided to study if melatonin can influence the antiproliferative effects of docetaxel in MCF-7 cells. Unfortunately we could not use the pharmacological doses of docetaxel (similar to those used in clinic) since docetaxel 1 μM kills 80% of MCF-7 after three days of treatment. Longer times of exposition to docetaxel result in non-survival of the cells. Therefore, we decide to test subpharmacological doses of docetaxel to address the effect of melatonin combined with

the taxane. The doses chosen were 1 nM and 0.1 nM; Physiological concentrations of melatonin potentiated the inhibition of proliferation caused by docetaxel. Interestingly, pretreatment of the cells with melatonin 24 hours in advance to docetaxel treatments resulted in a higher decrease of cell proliferation. These results point to melatonin as an enhancer of docetaxel antiproliferative effects when the taxane is used at subpharmacological doses. These results need to be subject to further research since they might have important clinical implications: if the same inhibitory effects of melatonin when administered together with docetaxel are obtained in clinica tests, that result might have potential applications. Patients receiving docetaxel as chemotherapy might benefit in the next future of a cotreatment with melatonin as an adjuvant agent, allowing a reduction in the dose of the chemotherapy agent administered, which might result in a better tolerance of the compound with less adverse effects.

Once established that melatonin enhanced the antiproliferative effects of subpharmacological doses of docetaxel, we next decide to examine whether the inhibition of proliferation was related to induction of apoptotic cell death.

For this purpose, we use an Annexin V-FITC detection kit which allow to identify among viable cells, early apoptotic cells, late apoptotic cells and necrotic cells. Again, cells were treated with subpharmacological doses of docetaxel in the presence or absence of melatonin. Once more, the pretreatment with melatonin was tested, adding melatonin to the media 24 h in advance of docetaxel. The taxane induced a significant decrease of the number of cells alive, increasing the percentage of cells in early apoptosis. In a parallel result to that obtained when proliferation was addressed, melatonin cotreatment enhanced the apoptotic effect of docetaxel, proving the pineal gland hormone to be more effective when added prior to the treatment of the taxane.

In summary, to date it has been well established that melatonin is an oncostatic and antiestrogenic compound effective when tested in estrogen-responsive cell-lines *in vitro* and also *in vivo* in animal models bearing estrogen-dependent mammary tumors. Melatonin is a molecule with high expectations of being considered as an interesting anticancer drug in the prevention and treatment of, not only estrogen-dependent tumors, but also, other kinds of cancer, since inhibitory effects have been demonstrated in gastric, lung, pancreatic or hematopoietic cancers. However, in the next years, much more additional research needs to be done to clarify if melatonin administration in combination with chemotherapy agents might constitute a new anticancer treatment. In particular, we think that future research concerning the role of melatonin as a non-toxic and low cost drug to be considered in breast and other types of tumors must be done, especially at molecular levels (**30**).

Systematic screenings addressing the effects of chemotherapy over the genes known to be altered in the different types of cancer, and how melatonin can modulate the expression and activity of

those genes, either when acting alone or in combination with chemotherapy. Once more, larger clinical trials need to be designed, and many more molecular studies (including gene expression profiles, post-translational modifications and individual gene tests) need to be done. With the data we handle at the moment, and considering that melatonin is a natural hormone with no adverse effects described to date, we believe it will be reasonable to strongly recommend melatonin as a potential drug to be considered in the treatment of estrogen-responsive breast cancer.

6.- Conclusions and further research

- Docetaxel induces changes in gene expression of transcription factors, tumor suppressor genes, adhesion molecules, proapoptotic and antiapoptotic genes in MCF-7 cells. Melatonin seems to modulate those changes accordingly with its previous well established oncostatic and antitumoral actions.
- Docetaxel inhibits proliferation of MCF-7 cells in a dose dependent manner. Melatonin enhances the antiproliferative effects of subpharmacological doses of this agent.
- Melatonin enhances the pro-apoptotic effect of docetaxel in MCF-7 cells, improving the killing efficacy of this chemotherapeutic agent.
- In summary, melatonin may benefit breast cancer patients who are receiving docetaxel and might have a potential to be an excellent adjuvant for chemotherapy treatments currently used in breast cancer.

According to this, the further research will be focused on:

- To test the modulatory effect of melatonin over the docetaxel altered expression of other genes not yet individually tested.
- To search for posttranslational modifications (cell-signaling pathways) triggered by docetaxel, characterizing the possible role of melatonin as a modulator of those changes.
- To screen the changes in gene expression, proliferation and posttranslational modifications induced by other chemotherapy agents used in breast cancer treatment, such as vinorelbine, doxorubicin or trastuzumab, in presence or absence of melatonin.
- To screen the changes in gene expression, proliferation and posttranslational modifications induced by radiotherapy, checking the effect of melatonin modulating those changes.

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