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Review

# Estrogen-signaling pathway: A link between breast cancer and melatonin oncostatic actions

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**Abstract**

*Background*: Melatonin exerts oncostatic effects on different kinds of tumors, especially on endocrine-responsive breast cancer. The most common conclusion is that melatonin reduces the incidence and growth of chemically induced mammary tumors, in vivo, and inhibits the proliferation and metastatic behavior of human breast cancer cells, in vitro. Both studies support the hypothesis that melatonin oncostatic actions on hormone-dependent mammary tumors are mainly based on its anti-estrogenic actions. *Methods and results*: Two different mechanisms have been proposed to explain how melatonin reduces the development of breast cancer throughout its interactions with the estrogen-signaling pathways: (a) the indirect neuroendocrine mechanism which includes the melatonin down-regulation of the hypothalamic–pituitary reproductive axis and the consequent reduction of circulating levels of gonadal estrogens and (b) direct melatonin actions at tumor cell level. Melatonin's direct effect on mammary tumor cells is that it interferes with the activation of the estrogen receptor, thus behaving as a selective estrogen receptor modulator. Melatonin also regulates the activity of the aromatases, the enzymes responsible for the local synthesis of estrogens, thus behaving as a selective estrogen enzyme modulator. *Conclusions*: The same molecule has both properties to selectively neutralize the effects of estrogens on the breast and the local biosynthesis of estrogens from androgens, one of the main objectives of recent antitumor pharmacological therapeutic strategies. It is these action mechanisms that 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.


*Keywords:* Melatonin; Pineal gland; Breast cancer; Estradiol; Estradiol receptor; Aromatase; MCF-7; Aromatases; Signaling pathways; Gap junction; Cell proliferation; Metastasis; Carcinogenic effects; Postmenopausal women; Mammary carcinogen; Steroid hormones; Oxidative damage; Antioxidant; Antiproliferative effect; Oncostatic actions; Immunomodulator; Cytochrome P-450; Pineal function; Ovarian function; Hypothalmus; Prolactic; E-cadherin expression; Cadmodulin; Indolamine

## 1. Introduction

The role of melatonin in tumor development has been under intensive study during the last few decades [1–3]. The oncostatic actions of melatonin have been studied on several kinds of tumors and especially on hormone-dependent breast cancer. There is a general agreement that melatonin, in vivo, prevents the promotion and growth of spontaneous or chemically induced mammary tumors in rodents, whereas in vitro melatonin inhibits breast cancer cell proliferation and invasiveness [1–4].

The mechanisms through which melatonin exerts its oncostatic properties (Fig. 1) are explained in a variety of ways based on the different known actions of this indolamine: (a) as indirect effects derived from the interaction of melatonin with the neuroendocrine reproductive axis [5] leading to a down-regulation of some of the hormones influencing tumor growth, especially gonadal estrogens; (b) as a consequence of melatonin anti-estrogenic actions on the epithelial mammary cells [6,7]; (c) as a consequence of its anti-oxidant properties [9]; (d) dependent on its immuno-enhancing effects [8]; or (e)

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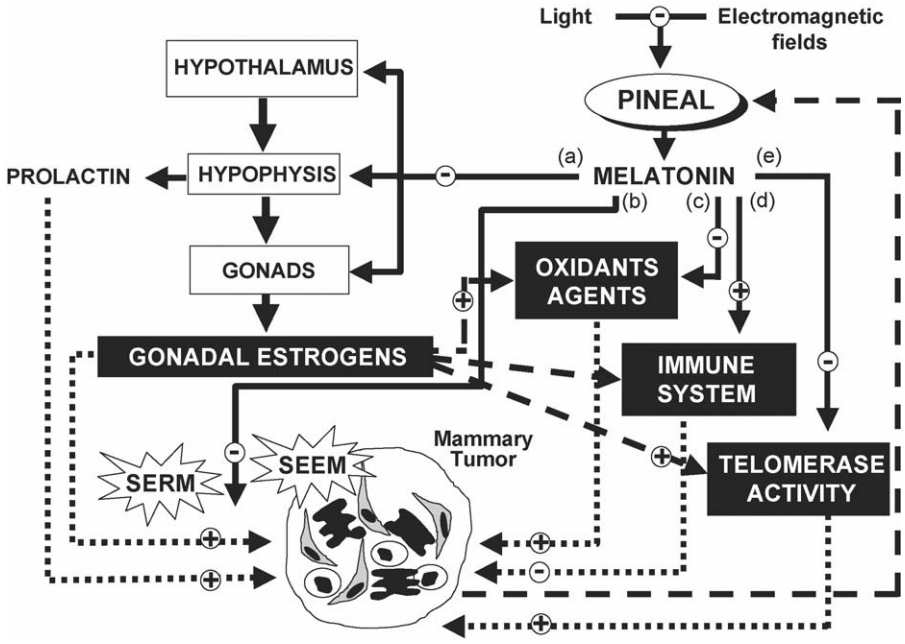


Fig. 1. Diagram that shows the mechanisms through which melatonin may modulate mammary carcinogenesis: (a) indirect effects on the neuroendocrine reproductive axis; (b) anti-estrogenic actions on the breast cancer cells, melatonin thus behaving as a selective estrogen receptor modulator (SERM) and a selective estrogen enzyme modulator (SEEM); (c) anti-oxidative effects; (d) immunomodulatory actions; or (e) inhibition of telomerase activity.

derived from its inhibitory effects on telomerase activity in tumor cells [10]. Of all these different mechanisms, by which melatonin may influence mammary cancer, we are going to focus on its interaction with estrogen-signaling pathways. Nevertheless, either directly or indirectly, melatonin–estrogen interaction is involved in all the above proposed oncostatic mechanisms of melatonin, since telomerase activity and immune function are, in some way, related to the levels of estrogens, and some estrogen metabolites are among the agents responsible for DNA oxidative damage.

The purpose of this review is to state how the estrogen-signaling pathway is one of the main links between breast cancer and melatonin oncostatic actions. In this review, we will firstly point out the importance of estrogens in the genesis and development of breast cancer; secondly, we will underline how the interaction between estrogens and melatonin has long been described to explain the melatonin action and, finally, we will outline the possible mechanisms through which melatonin may inhibit the growth of breast cancer and how these mechanisms involve interactions with the tumor cell estrogen-response pathway, with melatonin behaving as an antitumor drug with anti-estrogenic and anti-aromatase actions. We have selected the main results and conclusions obtained from the papers published in the last few decades which deal with the association of melatonin and breast cancer.

## 2. Breast cancer and estrogens

Breast cancer is the most frequent cancer in women (22% of all cancers in 2000) and is actually the second leading cause of cancer death among women after lung cancer (15% of cancer deaths). The estimated annual incidence of breast cancer worldwide is about one million cases [11]. Breast cancer is a classical model of hormone-dependent malignancy. It is known that estrogens are involved in the growth and differentiation of the normal mammary gland and they intervene in the growth of the ductal branching. However, there is considerable evidence that estrogens are also mammary carcinogens [12]. The role of ovarian estrogens in mammary cancer has been known since 1896, when Beatson observed beneficial responses of advanced breast cancer patients after bilateral ovariectomy. From then on, clinical and experimental studies have supported the idea that estrogen deprivation reduces mammary tumors and estrogen administration stimulates breast cancer growth [13]. The importance given to the role of estrogens in the genesis and growth of breast cancer has caused many factors (early age of menarche, late age of menopause, nulliparity, obesity, and hormonal usage after menopause) considered risk factors for breast cancer to be directly related with estrogens [11]. A common thread linking these factors is cumulative, excessive lifetime exposure to estrogen, suggesting that this exposure has an important role in the cause of breast cancer [11].

At this moment, although it is accepted that estradiol plays a main role in mammary tumorigenesis there is controversy over whether these effects are dependent on the stimulatory actions of estrogens on epithelial cell proliferation (indirect carcinogenic effects), or whether estrogens or their metabolites act as mutagenic agents (direct carcinogenic effects) [14,15].

In relation to the role of estrogens in the genesis and growth of mammary tumors, it is important to consider that two-thirds of breast cancers occur in postmenopausal women, where ovaries have ceased to be functional and circulating levels of estrogens are low. In premenopausal women, 70% of estrogens come from the ovaries and 30% from the conversion of androgens (mainly dehydroepiandrosterone and dehydroepiandrosterone-sulphate) to estrogens in peripheral tissues. After the cessation of the follicular function, in postmenopausal women, 100% of sex estrogens are synthesized in peripheral tissues from precursors of adrenal origin except for a small contribution from ovarian and/or adrenal testosterone and androstenedione. In this situation, plasma levels of estrogens are low and, however, the concentration of estradiol in peripheral tissues, such as the mammary gland, is high as a result of the in situ biosynthesis [16,17].

Because of the role of estrogens as a mammary carcinogen, one of the main objectives in the treatment of breast cancer has always been to neutralize the effects of estrogens on the breast. The ovariectomy, as mentioned above, was the first ''anti-estrogenic'' therapy. Since then, the treatment of breast cancer has included localized treatment on the breast region (surgery and radiotherapy) and general treatment that centers on chemotherapy and hormonotherapy. The pharmacological strategies employed to selectively neutralize the effects of estrogens on the breast are: (a) drugs that act through the estrogen receptor interfering with the effects of the endogenous estrogens; this group includes the so-called selective estrogen receptor modulators (SERMs) of which tamoxifen and its derivatives are the most representative examples and (b) drugs that interfere with the synthesis of steroid hormones by inhibiting the enzymes controlling the interconversion from androgenic precursors; these are selective estrogen enzyme modulators (SEEMs), which include steroidal (formestane, exemestane, etc.) as well as non-steroidal (anastrozole, letrozole, etc.) compounds [18]. The aim of this review is to explain that, among melatonin's many actions, there is one very important one, which is that it behaves as an oncostatic agent, in particular on estrogen-dependent tumors like breast cancer, and this melatonin antitumor action is going to be of anti-estrogenic characteristics. Melatonin shares properties with drugs of the two main pharmacological groups of substances employed in breast cancer treatment. This indolamine will in fact behave both as a SERM and as a SEEM.

## 3. Melatonin, the main pineal hormone

Melatonin is the main biologically active substance secreted by the pineal gland, an organ long considered by many scientists as a vestigial organ, until Lerner et al. [19] in 1958 isolated a substance, the *N*-acetyl-5-methoxytryptamine, which was called melatonin. Melatonin synthesis from tryptophan is made in successive steps involving different enzymes. The rate-limiting step in this biosynthetic pathway is the activity of the enzyme arylalkylamine *N*-acetyltransferase (NAT), which catalyzes the transformation of serotonin to *N*-acetylserotonin, the direct precursor of melatonin, and is controlled by the light–dark cycle. The regulation of the NAT activity depends on the suprachiasmatic nucleus of the hypothalamus which projects axons to the paraventricular nucleus of hypothalamus and from there to the thoracic spinal cord, where the axons synapses with preganglionic sympathetic neurons. These neurons project to the superior cervical ganglion, which sends postganglionic terminals to the pineal gland. The release of norepinephrine from these sympathetic terminals induces the activity of the NAT and promotes the synthesis of melatonin. The neurons of the suprachiasmatic nucleus act as an endogenous oscillator with period lengths close to 24 h, which is synchronized by the light–dark signals perceived by the eyes and transmitted by the retinohypothalamic tract. The light–dark cycle thus entrains the rhythm of melatonin synthesis. Circulating levels of melatonin are low during the day and show a large increase at night. The nocturnal rise of melatonin is abolished, or substantially decreased, in animals exposed to constant light whereas, in animals kept in constant darkness, melatonin is secreted following the endogenous rhythm driven by the suprachiasmatic nucleus of the hypothalamus [5,20]. The light–dark cycle is the main time factor of the regulating system of melatonin secretion and $\beta_1$-adrenergic blockers suppress the nocturnal melatonin secretion [5,20].

The actions of melatonin depend on its binding to specific receptors in the target tissues. Two melatonin receptors, termed MT-1 and MT-2, have been described in the central nervous system and in numerous peripheral tissues [20]. They are Gi-protein coupled membrane receptors that decrease intracellular concentration of cAMP [20]. Melatonin effects on other second messengers, such as $[Ca^{2+}]_I$, cGMP, diacylglycerol or protein kinase C, have been described depending on the cell type studied [20]. Although controversial, some reports based on the lipophilic nature of melatonin have also suggested that this hormone is able to bind and activate nuclear receptor members of the family of retinoid orphan nuclear receptors (RZR/ROR$_\alpha$) [21]. It has not, though, been possible to subsequently confirm this mechanism of action. Alternative mechanisms of melatonin actions, not mediated by receptors, have been also proposed. Melatonin may act at intracellular sites, binding to cytosolic calmodulin and thus affecting the calcium signaling and modulating cytoskeletal structural proteins [22]. Finally, recent in vivo and in vitro studies have shown that melatonin is a potent scavenger of hydroxyl radicals and other oxygen centered free radicals, protecting cells against oxidative damage [9].

Different physiological actions, in a great variety of biological contexts, have been attributed to melatonin. In mammals, it is involved in the synchronization of circadian

rhythms as well as in seasonal reproduction [5,23]. A considerable number of reports have appeared describing the effects of melatonin on the immune system, which suggests that melatonin has immunomodulatory actions, with high melatonin levels promoting and low levels suppressing a number of immune system parameters [8]. The detection of melatonin receptors in various lymphoid organs and in lymphocytes suggests multiple mechanisms of action. It has been proposed a dual role of melatonin as an immunomodulator: one is the activation of the immune system following an acute challenge (bacterial or viral infection) by potentiation of Th1 cell activity, macrophage function, and cytokine production; the other is a long-term resetting of the circadian modulation of immune functions, affecting hematopoiesis and thymocyte mitosis [8]. Melatonin has also been described as an efficient free radical scavenger with antioxidant properties [9].

## 4. Melatonin and estrogens

Melatonin was formerly considered as a hormone controlling seasonal reproduction in wild animals through its effects on the hypothalamic–pituitary reproductive axis [5,23]. In seasonally breeding mammals, melatonin controls the reproductive function through the activation of receptor sites within the hypothalamic–pituitary axis thus driving the levels of gonadal activity [24,25]. Melatonin down-regulation of the ovarian estrogen secretion has been observed in a variety of mammals (the so called long breeders) [5] whereas in other, such as sheep (short breeders), control of reproduction depends on the melatonin-induced up-regulation of the synthesis of estrogens during the winter [26]. In humans the role of melatonin on the reproductive system is not completely clear and even controversial. An inverse relationship between serum melatonin and ovarian activity [27] and a certain role of melatonin in the modulation of the neuroendocrine reproductive axis has been proposed [28]. Furthermore, direct stimulatory effects of melatonin on ovarian steroidogenesis have been demonstrated in human granulosa-luteal cells [29]. However, some other reports indicate no effect of melatonin on estrogens production by granulosa cells [30]. Together, these data suggest that melatonin modulates ovarian function although, in humans, this control on gonadal activity has not been solidly demonstrated. Based on the role of the pineal gland in inhibiting gonadal maduration and sex hormone secretion in mammals, Cohen et al. [31] proposed a possible relationship between the pineal function and the etiology of breast cancer and introduced, for the first time, the hypothesis that diminished function of the pineal gland may promote the development of breast cancer in human beings. They supported their hypothesis by indirect data, such as a strong correlation between pineal calcification (interpreted as diminished pineal function) and the incidence of breast cancer and a low incidence of breast cancer in psychiatric patients taking chlorpromazine, which raises serum melatonin. The authors suggested that a decrease in pineal function, whatever its cause, decreases melatonin levels and induces a relative ''hyperestrogenism'', which underlies the development of breast cancer in human beings [31]. Four years later, Tamarkin et al. [32] stated that the amplitude of the night time peak of plasma melatonin is diminished in women with estrogen receptor-positive breast cancer versus those with estrogen receptor-negative disease or healthy, matched controls [32]. Whether the decline in the nocturnal peak of plasma melatonin represents a long-term pineal failure or a change that occurs at the time of breast cancer development is not clear. Later, an inverse correlation between nuclear grade and tumor melatonin concentration and a positive association between tumor melatonin and estrogen receptor status suggested that high tumor melatonin concentration may reflect the differentiation state and constitute a good prognostic marker [33]. Pineal melatonin secretion may be modified in quantity as well as in rhythmicity in breast cancer patients [34].

Besides clinical and epidemiological studies, there is evidence from in vivo studies on animal models that supports the hypothesis that melatonin oncostatic actions on hormone-dependent mammary tumors are based on anti-estrogenic actions. Most in vivo studies have used, as an animal model, the chemically induced (7,12-dimethylbenz(*a*)anthracene or *N*-nitrosomethylurea) mammary cancer in rats. In spite of the great diversity of experimental approaches undertaken by the different groups involved in this research, what they all have in common is that they are based on a comparison between the effects of the carcinogen in animals with increased pineal function obtained by subjecting the animals to experimental manipulations known as enhancers of melatonin actions, by exposing the animals to short photoperiods which increase melatonin levels or by directly administering melatonin, and thus affects animals with something equivalent to decreased or suppressed pineal function obtained by surgical pinealectomy or exposure to very long photoperiods or continuous light. From these kinds of experiments, the general conclusions are that the animals with enhanced pineal function or those treated with melatonin, in contrast to pinealectomized animals or to those animals with decreased melatonin levels, have: (a) an increase in tumor latency or time elapsing between the administration of the carcinogen and the appearance of palpable mammary tumors, (b) a lower tumor incidence, (c) a reduction in the number and size of the tumors, (d) a lower rate of tumor growth, (e) a more frequent incidence of tumor regression in previously induced tumors, (f) a decrease in serum estradiol and pituitary follicular stimulating hormone (FSH) and luteinizing hormone (LH), and (g) a decrease in the estrogen receptor concentration at tumor level [1,2]. At first, these oncostatic effects of melatonin in vivo were explained by the down-regulatory effects of this indolamine on the neuroendocrine reproductive axis [5,35], and the consequent

reduction of hormones, such as estradiol and prolactin, which are responsible for the normal and pathological growth of the mammary gland. Besides the modulation of serum estrogens by melatonin, others possibilities were suggested to explain the melatonin oncostatic actions. Several years ago, our group demonstrated that the growth of chemically induced estrogen receptor-positive mammary tumors in ovariectomized rats treated daily with exogenous estradiol was significantly reduced when these animals were subjected to some of the experimental manipulations known as enhancers of pineal-dependent effects (anosmia, underfeeding or exposure to cold, associated with light deprivation) [1,2]. These animals subjected to increased pineal function, which had lower tumor growth than control and pinealectomized animals, also showed a reduction in the concentration of the estrogen receptor at the tumor level (Fig. 2). In this case, these antitumor effects could not be explained by a pineal-dependent decline in circulating estrogens, since serum estradiol concentrations were kept stable because of the exogenous administration of steroids and the lack of changes in the rate of metabolism of steroids. The results of these experiments and other similar ones suggested that melatonin may directly counteract the effects of estrogens at tumor level. This fact marked the beginning of the study of melatonin oncostatic actions in vitro directly on the tumor cell. At mammary tumor cell level, melatonin will interfere with the estrogen receptor and will counteract the effects of estrogens, thus behaving as a SERM, and, it will also regulate the activity of some enzymes controlling the local synthesis of estrogens, thus behaving as a SEEM. In the same molecule, we are going to have both properties to selectively neutralize the effects of estrogens on the breast.

### *4.1. Melatonin as a selective estrogen receptor modulator*

The direct anti-estrogenic effects of melatonin on breast cancer cells were evidenced from in vitro studies [1–4]. Most of them were carried out on a MCF-7 human breast cancer cell line, which was derived from the pleural effusion of a woman with metastatic carcinoma of the breast [36]. This mammary tumor cell line represents a good model for the study of the molecular mechanisms underlying estrogen action in breast cancer since it contains the estrogen receptors, $ER_\alpha$ and $ER_\beta$, and its growth depends on the presence of estrogens in the culture media [37,38]. The levels of some RNAs and proteins in these cells are also controlled by estrogens [39]. Recently, the expression of MT-1 melatonin receptors in these cells has been demonstrated [40,41].

In these estrogen-responsive MCF-7 cells, melatonin exerts a direct antiproliferative effect, including a decrease in cell number, DNA content and thymidine incorporation [42–44]. This antiproliferative effect has some important characteristics, such as: (a) it is reversible and when melatonin is removed from the culture, the cells recover their normal rhythm of growth; (b) it is specific to the melatonin molecule and the inhibitory effect is not shared with melatonin precursors, metabolites or other pineal methoxyindoles; (c) it is dose-dependent and only melatonin concentrations close to 1 nM (concentrations similar to those found in the serum of most mammals during the nocturnal period) are effective for decreasing cell proliferation, whereas supra- or sub-physiological ones lack these antiproliferative effects; (d) it is dependent on the presence

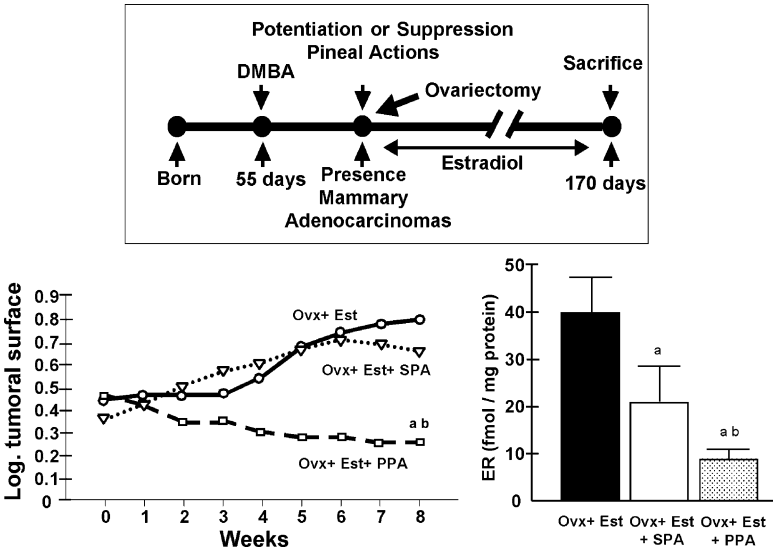


Fig. 2. (A) Time course of changes in tumor size. (B) Estrogen receptor concentration in mammary tumors. Rats bearing DMBA-induced mammary adenocarcinomas of 1 cm diameter were ovariectomized (Ovx) and subjected to manipulations either to potentiate (PPA) or suppress (SPA) pineal actions and their influence on the evolution of tumoral size evaluated weekly. Animals also received daily injections of estradiol (Est) or the diluent. The S.E.M. (never > 8%) have been omitted to clarify the graph. Differences between treatments at 8 weeks of experiment were: vs. (a) Ovx + Est ($p < 0.01$), vs. (b) Ovx + Est + SPA ($p < 0.01$). Modified from Cos and Sánchez-Barceló [2].

of estrogen receptors in the cells and, as well as in MCF-7 cells, melatonin inhibits proliferation in other estrogen-responsive cell lines (T47D, ZR-75-1), but has no effect on estrogen-insensitive breast tumor cell lines (BT-20, MDA-MB-231, MDA-MB-364, Hs587t, and $T47D_{DO}$); (e) it is dependent on the rate of cell growth, where the greater the rate of cell proliferation is the higher the level of the melatonin antiproliferative actions is; and (f) it is dependent on the presence of complete serum in the culture medium and in serum-free media melatonin loses its antimitogenic capabilities unless MCF-7 are also simultaneously exposed to some mitogen, such as estradiol, prolactin or epidermal growth factor, which supports the hypothesis that this indole is in some way interacting with a mitogen(s) present in the serum to inhibit cell proliferation [42,45]. The fact that only human breast cancer cell lines that express estrogen receptors have been found to be 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 [43]. The link between the antiproliferative effect of melatonin on MCF-7 cells growth and the estrogen-response pathway is further supported: (a) by the ability of melatonin to block, under different culture systems (monolayer and clonogenic soft agar) the mitogenic effect of estradiol [1,7,42]; (b) by the melatonin blockade of the estrogen-rescue of tamoxifen-inhibited cells in clonogenic agar and monolayer culture [7]; (c) by the ability of tamoxifen, a non-steroidal anti-estrogen, to reduce the antiproliferative action of melatonin, which suggests that both tamoxifen and melatonin may be working through the same or related mechanisms [43]; (d) by melatonin's down-regulation of both $ER_\alpha$ protein and $ER_\alpha$ mRNA expression in a time and dose-dependent manner in MCF-7 cells [6,46]; (e) by the melatonin modulation of estrogen-regulated proteins, growth factors and proto-oncogenes ($TGF_\alpha$, *c-myc*, pS2, progesterone receptor, *c-fox*, and $TGF_\beta$) in human breast cancer cells [45,47]; (f) by the enhanced growth suppression effects of melatonin in $MT_1$-transfected $ER_\alpha$-positive cells in comparison to parental and vector-transfected MCF-7 cells but, however, $MT_1$-overexpression did not induce a melatonin-sensitive phenotype in $ER_\alpha$-negative breast cancer cells [40]; and (g) by the enhanced inhibition of $ER_\alpha$ mRNA expression induced by melatonin in $MT_1$-transfected MCF-7 cells [40].

The interaction between melatonin and estradiol also takes place through regulatory effects on cell cycle kinetics (Fig. 3). Estradiol stimulates cell proliferation and induces the cell progression arrested in the $G_1$–S phase, whereas tamoxifen, a non-steroidal anti-estrogen used in the treatment of breast cancer, inhibits the proliferation of estrogen receptor-positive human breast cancer cells by arresting cell growth during the early part of the $G_1$ phase of the cell cycle and thus causing a transition delay into the S phase [48]. The antiproliferative effect of melatonin, like tamoxifen, is cell cycle specific by causing a $G_1$–S transition delay. The incubation with melatonin increased the fraction

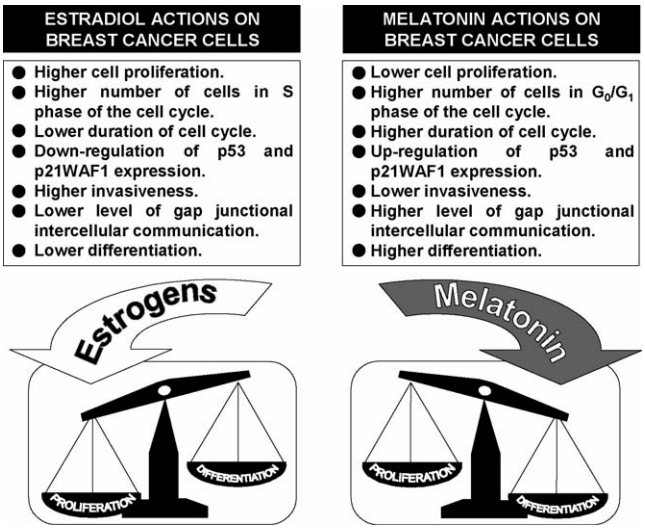


Fig. 3. The interaction between melatonin and estradiol takes place through regulatory effects on the balance between proliferation and differentiation. Estradiol stimulates the proliferation and reduces the differentiation. Melatonin shifts the balance in the opposite direction from estradiol, by promoting the differentiation and by inhibiting the proliferation of mammary tumor cells.

of cells in $G_0/G_1$ phase of the cell cycle while simultaneously causing a 50% reduction in the proportion of cells in S phase [7]. Melatonin has been described as being able to temporarily increase the duration of the cell cycle by more than three hours (15% of the total time) and then arrest cell progression in the cell cycle and/or delay the recruitment of some quiescent or non-proliferative cells [49]. Melatonin exerts part of its antitumor effect through a cell cycle specific mechanism opposite to estradiol. In addition, melatonin also decreases DNA synthesis in the cells that have progressed to the S phase of the cell cycle [44].

Changes in cell cycle kinetics always involve changes in some regulatory proteins of the cell cycle. The modulatory effect of melatonin on the cell cycle can be explained by the effects of this indolamine on the expression of some of the proteins involved in the control of the $G_1$–S transition. Thus, it has been demonstrated that melatonin, at nanomolar concentrations, increases the expression of p53 and p21WAF1 [50]. The p53 is a protein that activates the expression of the p21WAF1 gene that inhibits cyclin-dependent kinases thus leading to a failure of the phosphorylation of the retinoblastoma protein and the subsequent blockade of cell progression from $G_1$ to S. The up-regulation of the expression of p53 and p21WAF1 proteins induced by melatonin has been suggested as an important mechanism by which melatonin causes a cell cycle transition delay at the $G_1$–S interface. Thus, the accumulation of cells in the $G_1$ phase induces them to enter the $G_0$ phase, a quiescent state that leads the cells to a higher differentiation. This suggests that the antiproliferative effects of melatonin on MCF-7 human breast cancer cells may be due in part to its ability to shift the balance from proliferation to differentiation (Fig. 3). It has been long known that proliferation and

differentiation appear to have a reciprocal relationship [51]. Estradiol stimulates the proliferation of tumor cells, induces the progression of cells in the cell cycle and provides a lower opportunity for them to differentiate. Melatonin appears to shift the balance in the opposite direction from estradiol, by promoting the differentiation and by inhibiting the proliferation of mammary tumor cells. Morphological and morphometric studies have shown that physiological concentrations of melatonin allow human breast cancer cells to achieve ultrastructural features typical of terminally differentiated cells (smaller cells, presence of cytokeratin filament bundles, conspicuous rough endoplasmic reticulum and *Golgi cisternae*, prominent nucleoli at the nuclear level) and these ultrastructural changes are counteracted by estrogens [52].

This increase of tumor cell differentiation induced by melatonin also extends to the metastatic behavior of MCF-7 cells. It is well known that estradiol stimulates the invasive and the metastatic potential of cancer cells, increasing the ability of MCF-7 cells both to form tumors and produce distant metastasis in the nude mice [48] and enhancing the ability of these cells in vitro to invade through an artificial reconstituted basement membrane [53]. In vitro, melatonin at physiological doses (1 nM) reduces the invasiveness of MCF-7 human breast cancer cells by decreasing their capacity for attachment to the basement membrane and by reducing their chemotactic response [54]. Melatonin is also able to block the 17β-estradiol-induced invasion [54]. As previously reported [53,54], estrogens induce a marked change in the interaction of the MCF-7 cells with basement membrane components; changes that are characteristic of the malignant phenotype. Estrogen-treated cells show a greater attachment to the basement membrane, a greater ability to migrate toward laminin, a greater proliferation in culture in the presence of basement membrane matrix and a much greater ability to invade the barriers of reconstituted basement membrane [53]. The presence of melatonin (1 nM) in the culture medium significantly reduces the ability of MCF-7 cells to attach to the basement membrane and also counteracts the stimulatory effects of 17β-estradiol on cell adhesion. Additionally, melatonin reduces the chemotactic response of MCF-7 cells and attenuates estradiol-induced cell migration [54]. Tumor cell motility and invasion are adhesion-dependent phenomena related with the presence of cell surface adhesion molecules for both cell–cell and cell–matrix interactions. A down-regulation or a loss of expression of some of these cell 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 process [55]. Physiological doses of melatonin increase the MCF-7 expression of $\beta_1$-integrin and E-cadherin, two important molecules for cell-to-cell and cell–matrix interactions, respectively, and then shift tumor cells to a lower invasive status by promoting the differentiation of tumor cells [54]. The in vitro anti-invasive effect of melatonin has been also correlated with an in vivo decrease of the tumorigenicity of MCF-7 cells induced by this indolamine. Melatonin reduces tumor formation, the size of tumors and the incidence of distant metastasis in ovariectomized athymic nude mice implanted with 17β-estradiol subcutaneous pellets and inoculated with MCF-7 cells [54].

Further evidence of the level of cell differentiation is the establishment and maintenance of the intercellular gap junctional contacts. Gap junctional intercellular communication is known to be involved in controlling cell proliferation and differentiation and plays a crucial role in the suppression of tumor promotion. Abnormalities in gap junctional intercellular communication have been observed in cancer cells. Most, if not all, cancer cells have some dysfunction in gap-junction-mediated intercellular communication, either because of defects in cell adhesion or their inability to have functional gap junctional communication. Growth factors are tumor promoters that inhibit gap junction function and several oncogenes linked to the control of cell proliferation and differentiation that affect gap junctional communication as a part of their oncogenic function [56]. In addition, most, if not all, tumor-promoting chemicals are able to down-regulate gap junction function, while some antitumor-promoting chemicals can up-regulate gap junctional communication [56]. This is the case of melatonin that modulates the levels of gap junctional intercellular communication in breast tumor cells. Melatonin (10 μM or 1 nM) increases the level of gap junctional intercellular communication in breast cancer cells, which would allow the transfer of molecules between adjacent epithelial cells that would regulate their growth [57]. Progressive loss of gap junctional intercellular communication and loss of E-cadherin have been observed to occur in advancing metastatic disease, which support the importance of functioning gap junctions and cell adhesion molecules to proper cell control [58]. Since melatonin increases E-cadherin expression [54], it has been postulated that, via this, it could be a possible mechanism through which melatonin acts as a differentiating agent and may increase cell-to-cell interactions and increase gap junctional intercellular communication in MCF-7 cells [57].

In the mammary gland, the role of melatonin as an agent that promotes the differentiation has been studied not only in mammary tumor cells but also in normal mammary cells that are sensitive to initiating a breast cancer [59]. In rodents, melatonin treatment decreases the development and the number of epithelial structures linked to sites of growth: terminal, lateral and alveolar buds, highly sensitive to tumor initiation by chemical carcinogens and, in contrast, it increases the number of the epithelial structures representing the final stage of ductal growth and which are more resistant to cancer initiation by exposure to carcinogens [59]. The occurrence of melatonin-induced reduction of the number of terminal end buds must be emphasized in view of the fact that the density of these undifferentiated structures in the mammary gland correlated positively with the incidence of chemically induced adenocarcinomas [12]. The possible

role of melatonin in preventing mammary gland carcinogenesis could be found in its ability to differentiate the mammary gland. Transgenic mice overexpressing the N-ras proto-oncogene under the transcriptional control of the MMTV-LTR develop hyperplasic alveolar nodules (premalignant lesions) with dysplastic epithelial cells that overexpress N-ras protein as well as mammary adenocarcinomas. The treatment of these transgenic mice with melatonin significantly reduces the incidence of these lesions and reduces the expression of N-ras providing further evidence for melatonin's differentiating action [60].

The mechanism involved in the anti-estrogenic actions of melatonin is still being studied. Unlike the ''classic'' anti-estrogens, such as tamoxifen and its derivates, melatonin neither binds to the estrogen receptor nor interferes with the binding of estrogens to their receptor [6,46,61]. What melatonin seems to do is to decrease the expression of $ER_\alpha$ and to inhibit the binding of the $E_2$–ER complex to the estrogen response element (ERE) on DNA [6,61]. These effects have been shown to be dependent on melatonin binding to specific melatonin (MT-1) membrane receptors and the overexpression of these receptors in MCF-7 cells enhances the response of these cells to the anti-estrogenic effects of melatonin [40,41]. Thus, melatonin behaves as an anti-estrogen that does not bind to the estrogen receptor but to its own membrane receptors, and via this binding to its specific receptors it is able to interact with the estrogen receptor-signaling pathway.

What are the links between the signaling pathways of melatonin and estrogens? A possible interplay between these two pathways could be the opposing modulation of cAMP intracellular concentrations. The $ER_\alpha$ may be activated by elevated intracellular concentrations of cAMP. In MCF-7 cells, estrogens activate adenylate cyclase increasing intracellular cAMP by a non-transcriptional mechanism that involves steroid-induced modulation of cytoplasmic, or cell membrane-bound regulatory proteins (non-genomic actions) [62]. The cAMP synergizes with the genomic actions of steroids since it enhances ER-mediated transcription [62]. Alternatively, melatonin, working through the membrane-bound Gi protein-coupled MT-1 receptor, inhibits adenylate cyclase activity and decreases cAMP [63]. A melatonin-induced reduction in cAMP could be a mechanism by which the indolamine decreases $E_2$-induced $ER_\alpha$ transcriptional activity. In this sense, it has been demonstrated that melatonin inhibits forskolin-induced and $E_2$-induced elevation of cAMP in MCF-7 cells and inhibits $ER_\alpha$ gene transcription [64].

Another possible link for melatonin-estradiol interaction may be calmodulin (CaM). The association of CaM with the $E_2$-ER complex facilitates its binding to an ERE, thus suggesting a role for CaM as a modulator of the transcriptional activity of the estrogen receptor. Interestingly, only $ER_\alpha$, but not $ER_\beta$, interacts with CaM stimulating the phosphorylation of the receptor, thus facilitating the binding of estrogen as well as that of the $E_2$–ER complex to the ERE [65]. In this context, melatonin is known to exert modulatory effects on the $Ca^{2+}$/CaM signaling pathway [66]. Melatonin binding to $Ca^{2+}$/CaM inactivates the complex, thus counteracting its positive effects on the estrogen-signaling pathway [61].

As indicated above, one of the desirable properties of a SERM is its ability to specifically block the $ER_\alpha$ but not $ER_\beta$. Recently, it was demonstrated that whereas melatonin is a specific inhibitor of $E_2$-induced $ER_\alpha$-mediated transcriptional activation, it does not inhibit $ER_\beta$-mediated transactivation [67]. The sensitivity of the MCF-7 human breast cancer cells to melatonin depends on the $ER_\alpha/ER_\beta$ ratio and is abolished by $ER_\beta$ overexpression [67].

### 4.2. Melatonin as a selective estrogen enzyme modulator

In the breast cancer of postmenopausal women, estrogens are synthesized in the mammary tissue by transformation from androgenic precursors of adrenal origin. Estrogens are the product of androgen metabolism catalyzed by the enzyme complex known as aromatase. This enzyme complex consists of two components: aromatase cytochrome P-450 protein and, coupled to it, a ubiquitous flavoprotein, NADPH-cytochrome P-450 reductase [68]. The gene coding for the cytochrome P-450 protein is the largest of the cytochrome P-450 family. Because its overall homology to other members of the P-450 superfamily is low, aromatase belongs to a separate gene family designated CYP19 [69]. The transcription of the aromatase gene is highly regulated and each tissue can regulate the amount of aromatase transcribed in a highly specific manner [68].

The expression of aromatase is highest in the stromal compartment of breast tumors but is also present in epithelial cells as well [69]. MCF-7 cells express aromatase and, then, they are able to synthesize estrogens from androgens and these estrogens synthesized in situ can exert their autocrine and paracrine action promoters of the cell proliferation binding to the estrogen receptor of the own tumor cell or adjacent tumor cells. Thus, the proliferation of MCF-7 cells increases in the presence of testosterone in the culture medium. MCF-7 cell proliferation induced by testosterone can be blocked in a dose-dependent manner by the simultaneous administration of tamoxifen and, on the other hand, is not affected by simultaneous addition of an inhibitor of the androgen receptor, which suggests that the stimulatory effects of testosterone on cell proliferation is not mediated via androgenic receptors, but via estrogenic receptors through its transformation into estrogens which bind to it.

Physiological concentrations of melatonin counteract the testosterone-induced cell proliferation dependent on the local biosynthesis of estrogens from testosterone by the aromatase activity of the cells. In particular, melatonin reduces the aromatase activity (measured by the tritiated water release assay) of MCF-7 cells both at basal conditions and when aromatase activity is stimulated by cAMP or

cortisol [70]. The greatest inhibition of the aromatase activity has been obtained with 1 nM melatonin, the same concentration that gives the highest antiproliferative and anti-invasive effects of MCF-7 cells. Finally, by RT-PCR, it has been shown that melatonin down-regulates aromatase mRNA steady state levels in MCF-7 cells [70].

This modulator effect of melatonin on the enzyme that controls the conversion from androgenic precursors to estrogens has been also described in vivo, in rats bearing DMBA-induced mammary tumors [71]. Since the growth of these tumors is estrogen-dependent, the ovariectomy significantly reduces the size and number of the tumors while the administration of testosterone to ovariectomized animals is able to maintain the tumor growth at the control animals level. The stimulatory effect of tumor development induced by testosterone, which depends on the local synthesis of estrogens due to the aromatase action, is suppressed by the administration of melatonin [71]. The growth-stimulatory effects of testosterone on the tumors depend exclusively on locally formed estrogens, since no changes in serum estradiol have been described in testosterone-treated rats. In tumors from animals treated with melatonin the lowest microsomal aromatase activity has been found, so melatonin could exert at least part of its antitumor effects on hormone-dependent mammary tumors by inhibiting the aromatase activity of the tumor tissue and then reducing the local synthesis of estrogens [71].

The aromatase gene (CYP19) is, in mammary cancer cells, under the control of promoters II and I.3, regulated by cAMP [72]. Melatonin, through a membrane-bound Gi protein-coupled receptor (MT-1) down-regulates cAMP in different cell types [63,64]. Since in MCF-7 cells, it has been demonstrated that melatonin at nanomolar concentration reduces the forskolin-induced increase of cAMP [64] and, in murine mammary tissue melatonin decreases cAMP and increases cGMP in a dose- and time-dependent way [73], it has been suggested that melatonin could modulate aromatase through its modulatory activity on cAMP.

## 5. Conclusions

Estrogens are involved in the growth and differentiation of the normal mammary gland and have an important role in the genesis and growth of breast cancer. Melatonin, the main secretory product of the pineal gland, acts as a regulator of neoplastic cell growth, particularly on those tumors corresponding to the mammary gland. Experimental manipulations activating the pineal gland, or the administration of melatonin, reduce the incidence and growth rate of

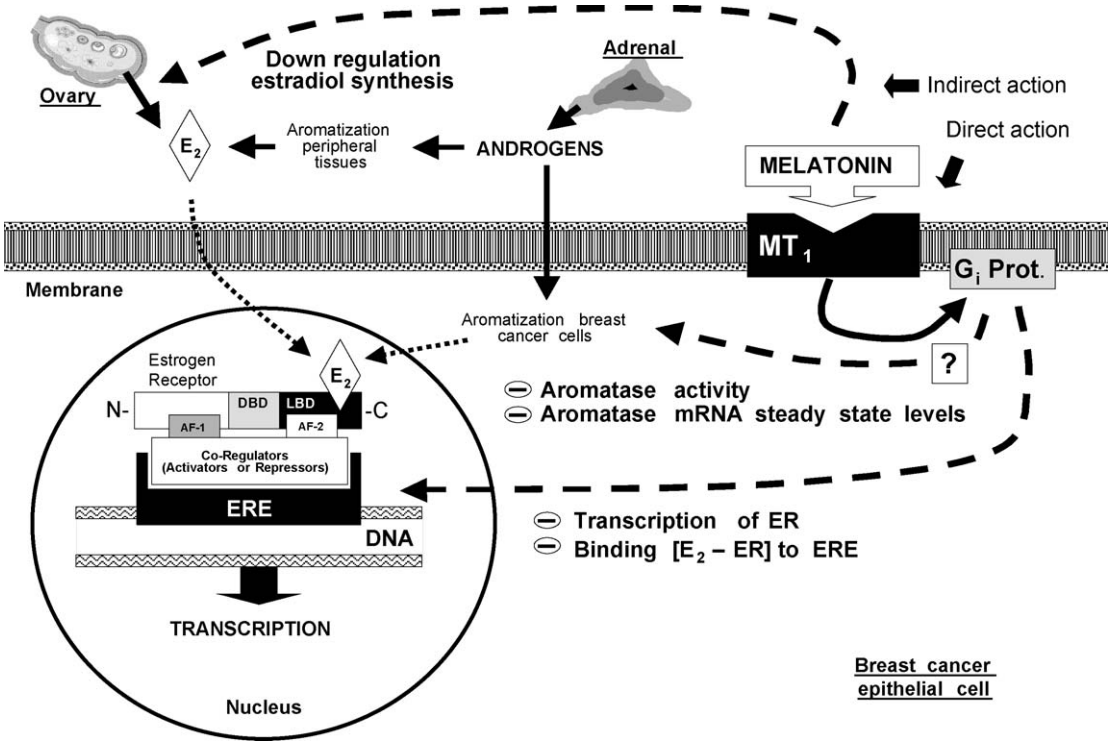


Fig. 4. Outline of the mechanisms whereby melatonin reduces the development of breast cancer mediated by estrogens: indirect mechanisms, such as the melatonin down-regulation of the neuroendocrine reproductive axis and the consequent reduction of estrogenic hormones, and direct melatonin actions on the tumor cell. The direct actions are based on interactions with the tumor cells' estrogen-response pathway, by decreasing the expression of estrogen receptor and inhibiting the binding of the estradiol–estrogen receptor complex ($E_2$–ER) to the estrogen response element (ERE) in the DNA, which effect depends on melatonin binding to specific membrane receptors (MT-1), thus behaving as a selective estrogen receptor modulator; and by reducing the activity and mRNA steady state levels of the aromatase, the enzyme responsible for the local synthesis of estrogens, thus behaving as a selective estrogen enzyme modulator. The binding of melatonin to the MT-1 receptor as mediator of its anti-aromatase effect is still unknown.

chemically induced mammary tumors in rodents, while pinealectomy or situations that implicate a reduction of melatonin production usually stimulate mammary carcinogenesis. Melatonin reduces the estrogen-mediated development of breast cancer on the basis of two different mechanisms (Fig. 4): indirect neuroendocrine mechanisms, such as the melatonin down-regulation of the neuroendocrine reproductive axis and the consequent reduction of estrogenic hormones responsible for the normal and pathological growth of the mammary gland, and, on the other hand, direct melatonin actions at the tumor cell level. Evidence from in vivo studies on animal models and in vitro studies on human breast cancer cell lines supports the hypothesis that melatonin oncostatic actions on hormone-dependent mammary tumors are mainly based on anti-estrogenic actions and they are due to melatonin interactions on the tumor cells estrogen-response pathway. At the level of the mammary tumor cell, melatonin will interact with the estrogen-response pathway and will counteract the effects of estrogens, thus behaving as a selective estrogen receptor modulator, and it will regulate the activity of the aromatase, the enzyme responsible for the local synthesis of estrogens, thus behaving as a selective estrogen enzyme modulator. The same molecule has both properties to selectively neutralize the effects of estrogens on the breast, one of the main objectives of the antitumor pharmacological therapeutics. These actions at different levels of the estrogen-signaling pathways, collectively, make melatonin an interesting anticancer drug in the prevention and treatment of estrogen-dependent mammary tumors.

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