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REVIEW

Molecular mechanisms of melatonin's inhibitory actions on breast cancers

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Abstract Melatonin is involved in many physiological functions and it plays an important role in many pathological processes as well. Melatonin has been shown to reduce the incidence of experimentally induced cancers and can significantly inhibit the growth of some human tumors, namely hormone-dependent cancers. The anticancer effects of melatonin have been observed in breast cancer, both in in vivo with models of chemically induced rat mammary tumors, and in vitro studies on human breast cancer cell lines. Melatonin acts at different physiological levels and its antitumoral properties are supported by a set of complex, different mechanisms of action, involving apoptosis activation, inhibition of proliferation, and cell differentiation.

Keywords Melatonin · Breast cancer · Epigenetic effects · Apoptosis · Growth inhibition · Cell morphology

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Introduction

In the last two decades, a compelling body of evidence has outlined the relevance of melatonin to human physiology and pathology. Melatonin displays important roles in several biologic functions, among which are circadian rhythms, sleep, mood, reproductive physiology, and diseases of aging [1–8]. Additionally, in the event of elevated oxidative stress, melatonin functions as a highly efficient antioxidant [9–12]. Numerous studies, based on animal as well as on clinical data, have provided evidence that melatonin reduces the incidence of experimentally induced cancers [13–15] and may significantly inhibit the growth of some human tumors [16–18]. The general conclusion is that melatonin inhibits cell proliferation and induces apoptosis in tumors (especially hormone-sensitive cancers), and decreases the incidence and the proliferation rate of chemically induced murine neoplasias. Interestingly, there is no consensus regarding the major mechanisms by which melatonin reduces tumor growth although numerous well-supported explanations have been proposed [19–24].

The first suggestion concerning a potential relationship between the pineal gland and cancer was made more than 80 years ago [25]. Soon after the discovery of melatonin, its role in the control of neoplastic growth quickly was often investigated [26, 27]. In 1978, the seminal paper by Cohen et al. [28] first proposed that the pineal gland and its main secretory product are likely to play an important role in the pathogenesis of breast cancer. These authors suggested that a reduction in pineal function, whatever its cause, and the consequent loss in melatonin secretion, may induce a state of relative hyperestrogenism, thus leading to a prolonged exposure of breast tissue to estrogens and eventually ending in cancer induction. Studies performed on the field in recent decades have highlighted that the

anticancer effects exerted by melatonin on breast tumors are complex and can be recognized at different physiological levels (from the molecular to the endocrine level) [21].

Breast cancer is one of the most frequently occurring cancers, and one of the leading causes of death among women aged 40–55 years [29]. Many factors, such as genetics, hormonal environment, age, diet, alcohol consumption, and cigarette smoking, have been hypothesized as contributors to the development of breast cancer [30–32]. However, a major consequence of a modern lifestyle is the disruption of circadian rhythms, a condition that leads to several pathological conditions, including sleep disturbances and depression [20, 33]. The alternation of the day and night circadian cycle is indeed a very important regulator of a wide variety of physiological biorhythms in organisms, including humans. In particular, accumulating evidence shows that alteration of circadian rhythms might lead to increased susceptibility to cancer in humans. Epidemiological studies have revealed the risk for breast cancer to be significantly higher in industrialized societies, and that the risk increases in women who work night shifts, and in individuals who spend more hours working at night [34]. Recently, experimental data has provided compelling evidence in support of such a hypothesis [35].

Growing in parallel with industrialization, the use of artificial light prolonged the "day," permitting employers to extend their work schedules well into the night and in many cases throughout the 24-h period. Because of this temporal coincidence, light at night suppresses the synthesis and release of melatonin; this drop has been incriminated as a plausible contributor to the elevated cancer risk [26, 36]. What this means is that humans in modern societies are rendering themselves progressively more melatonin-deficient by shortening their daily dark period, which also reduces the total amount of melatonin produced [24]. Kerenyi et al. [37] were early advocates of the idea that "light pollution" might be a potentially important etiologic influence on the genesis of other human cancers. Epidemiologic studies [38, 39] have shown that women working night shifts have a significantly elevated risk of breast cancer, which is likely related to circadian disruption, sleep deprivation, and melatonin suppression [33]. In 1981, Bartsch et al. [40] published an early study demonstrating that plasma concentrations are diminished in patients with breast cancer. Since then, other reports have confirmed that patients with established breast cancer have measurably lower levels of melatonin [41, 42]. Overall, these studies highlight how the nocturnal melatonin rhythm may represent a critically important chronobiotic signal, which not only directly inhibits human breast cancer signal transduction, but temporally organizes cancer metabolism and growth to help maintain the host–cancer balance. Disruption of tumor circadian organization shifts the host–cancer balance in favor of constantly up-regulated tumor metabolism and fuels "runaway" cancer cell proliferation and survival.

Melatonin's effects on breast cancer: animal studies and clinical trials

Insights into the relationship between melatonin and breast cancer have been provided by studies performed on chemically induced mammary cancer in animals. Reducing circulating melatonin levels in rats (through pinealectomy, or by exposure to different photoperiods), generally leads to increased spontaneous tumor induction or enhanced growth of implanted cancers [43]. Conversely, restoring melatonin levels generally prevents or restrains the development of breast cancer [44, 45].

Melatonin significantly reduces the incidence and tumor size of rat mammary cancers induced by 7,12-dimethylbenz[a]anthracene (DMBA) or *N*-nitrosomethylurea (NMU) [43, 46]. In DMBA-exposed rats, long-term daily administration of melatonin inhibited tumorigenesis, whereas pinealectomy increased the incidence of breast tumors [47]. Similar results have been reported by several authors [48]. Moreover, constant light (known to suppress melatonin release) reduces the latency and increases the number of DMBA-induced mammary tumors in rats; it also increases the incidence of different spontaneous cancers in female CBA mice [49]. Moreover, in NMU-treated rats, melatonin's cytostatic effects are similar to those exerted by tamoxifen, i.e., melatonin increases tumor latency, reduces cancer incidence (% of animals developing tumors) and also reduces the number and size of tumors [46]. Furthermore, melatonin retards the rate of tumor-growth and enhances spontaneous tumor regression [50, 51]. Collectively, these data show that melatonin inhibits both cancer initiation and progression, through several mechanisms, including estrogen-pathway modulation, receptor-mediated and receptor-independent effects on different enzymatic processes, as well as anti-oxidant effects [21]. It is well documented that reactive oxygen species (ROS) participate in a variety of processes regulating cell growth, gene transcription, differentiation, and apoptosis [52]. In cancer cells, free radicals and ROS can act as tumor promoters, leading to cancer initiation or to the growth enhancement of already-transformed cells. Therefore, free radical scavengers and antioxidant molecules like melatonin can display a significant role in preventing cancer and/or in hindering its progression [53, 54].

Some results obtained from research carried out on animals have been confirmed in studies performed in humans. In addition to some preliminary anecdotic reports

[55, 56], limited clinical trials carried out by Lissoni and colleagues [57–63] have provided evidence of benefits in treating cancer-bearing patients with melatonin. In a pilot study, 14 women with metastatic breast cancer who had no clinical response to tamoxifen alone were given 20 mg of tamoxifen plus 20 mg of melatonin in the evening. A partial response, defined as radiographic-confirmed reduction of lesions by greater than 50 %, was observed in four out of 14 patients (28.5 %) with a median duration of 8 months [57]. In two randomized clinical trials [58, 59] (including several types cancer, including breast tumors), melatonin had beneficial effects among patients with metastatic cancer.

Panzer and Viljoen [64] have reviewed clinical studies with melatonin on patients with different types of cancer, providing evidence about melatonin therapy benefits, and showing that the indoleamine, may: (a) increase survival in a few metastatic patients; (b) retard cancer progression; (c) improve quality of life and performance status; (d) decrease the incidence and severity of some side-effects linked to conventional treatments (hypotension, thrombocytopenia, myelodysplastic syndrome, lymphocytopenia) [61–63, 65]. In the evaluated reports, several types of advanced cancer patients are covered and only a few cases of mammary tumors; thus, little information on the potential efficacy of melatonin treatment in breast cancer patients is currently available. Additionally, these findings require verification by independent and controlled replication studies to overcome statistical bias and methodological deficiencies due to the limited number of patients under study [66].

Melatonin's effects on breast cancer: in vitro studies

Inhibition of breast cancer cell growth

The anticancer effects attributed to melatonin have often been observed in in vitro studies carried out on estrogen-responsive human breast cancer cell lines. The first such experiments using human MCF-7 cells demonstrated that melatonin, even at physiological concentrations, directly suppresses cancer cell growth [67]. Melatonin appears to exert an inhibitory effect by causing an accumulation of cells in the G_0/G_1 phase of the cell cycle [68] or, otherwise, by delaying the progression of MCF-7 cells from the G_1 phase to the S phase of the cell cycle [69, 70] allowing the cells to achieve a greater differentiation. A similar pattern was observed for other estrogen-sensitive cancer cell lines (T47D and ZR75-1) [71–73]. Growth-inhibition is accompanied by a significant reduction in DNA content and thymidine incorporation [74, 75]. These effects seem to be related to both cancer cell characteristics and culture conditions.

Melatonin receptors and estrogen receptors

Melatonin significantly inhibits cell growth in breast cancer cells expressing estrogen receptors (ERα) [76, 77]. Melatonin does not inhibit the proliferation of MDA-MB-231, MDA-MB-330, or BT-20 ERα-negative human breast tumor cells lines. However, the indoleamine has been demonstrated to reduce growth proliferation on ERα-negative breast cancer and progesterone receptor–negative human breast tumor xenografts growing in nude rats [78]. Moreover, a significant oncostatic action has been observed in ER-negative, non-breast tumors treated with melatonin [79]. These data suggest that some non-estrogen receptor-mediated effects are likely to be elicited by the complex interplay between melatonin and cellular molecules not related to estrogen receptors.

Certainly, some of the effects of the indoleamine are mediated by the interaction with a specific membrane-bound melatonin receptor. Numerous reports have demonstrated that melatonin binds and activates the G protein-coupled membrane receptors 1 (MT1) and 2 (MT2) in a variety of tissues [80, 81].

The oncostatic effects of melatonin on ER-positive breast cancer cells seem to be, at least in a major part, dependent on the presence of the MT1, which has been found in human breast cancer tissues [82]. The MT1 receptor is differentially expressed in ERα-positive and ERα-negative breast cancer cells, with the higher MT1 levels found in the former cell lines [83]. The MT1 receptor couples with different $G_{\alpha i}$ proteins in multiple cell types, while also coupling with the G_q and G_{11} proteins in other cell types [84]. Selective MT1 antagonists (i.e., luzindole) suppress melatonin-induced anticancer effects [84, 85] while, overexpression of MT1 receptor in MCF-7 cells significantly enhances the response of these cells to the growth-inhibitory actions of melatonin, both in vitro and in vivo [86, 87]. Similar results have been observed when treating MCF-7 cells with valproic acid, a MT1 receptor inducer [88]. The sensitivity of different MCF-7 strains to melatonin is strongly dependent on MT1 expression [89]. Significantly diminished night-time and early morning levels of MT1 receptors were observed in uteri from old rats compared to adult and young animals; in association with this reduction, the growth-suppressive action of exogenous melatonin was found to be diminished in old rats [90]. The MT2 receptor seems not to be involved in oncostatic effects triggered by melatonin, in that MT2 activation is incapable of mediating the antiproliferative effects of melatonin on breast tumors [91]. Recent findings also demonstrated that the MT1 receptor co-localizes with the Cav-1 antibody, indicating the MT1 receptor resides in the caveola, a key membrane-signaling platform [92].

MT1 and MT2 receptors are G-protein-coupled receptors, which are expressed in various parts of the central nervous system and in numerous peripheral organs (blood vessels, mammary gland, gastrointestinal tract, liver, kidney and bladder, ovary, testis, prostate, skin, and the immune system) [93]. Indeed, melatonin's receptor may exist in every cell in the body. Melatonin receptors mediate a plethora of intracellular events depending on the cellular milieu. These effects include changes in intracellular cyclic nucleotides (cAMP, cGMP) and calcium levels, activation of certain protein kinase C subtypes, intracellular localization of steroid hormone receptors, and regulation of G protein signaling proteins [81, 94].

MT1 expression is regulated by both melatonin and estradiol, as first documented in experiments performed on cells of the pars tuberalis [95]. The steady-state level of MT1 mRNA is significantly enhanced in MCF-7 cells cultured in estradiol-depleted medium. In cancer cells cultured in the presence of fetal bovine serum (FBS), the MT1 receptor steady-state mRNA level is suppressed by the addition of estradiol (1 nM) or significantly diminished by the addition of melatonin, confirming the ability of melatonin to down-regulate the levels of its own receptor, at least at the steady-state mRNA levels [91, 96]. The ability of estradiol to down-regulate MT1 receptors could explain some contradictory results, i.e., the lack of melatonin inhibition on estradiol-induced proliferation of breast cancer cells [97].

While removal of estradiol from the culture media up-regulates MT1 levels, several reports were unable to demonstrate an enhanced growth-inhibitory response to melatonin in MCF-7 cells growing in estradiol-deficient media, as the overall growth of those cells is generally slowed in the absence of estradiol [98]. These results imply that a number of other hormones, cytokines, or growth factor-related signaling pathways modulate MT1 expression, and the hormonal milieu of the tumor at the time of melatonin administration may dramatically impact the responsiveness of the tumor to the anti-proliferative action of melatonin. These actions are generally recognized as hormone-like effects. However, melatonin does not always act in this manner, and several melatonin-induced actions are carried out without the intervention of a receptor [98–100]. Melatonin should be rather considered as a tissue factor, behaving like a paracoid, an autocoid, an antioxidant, or a pro-oxidant factor depending on the physiological context [101].

The oncostatic effects triggered by melatonin are strictly context-dependent. Reducing the FBS concentration abrogates the responsiveness of MCF-7 cells to melatonin, until cells are totally refractory in serum-free medium [102]; on the contrary, melatonin-induced inhibition is enhanced in both human [76] and animal cancer cells [72] cultured in stripped serum supplemented with estradiol. Moreover, differences in MCF-7 cell strains and especially differences in their proliferation rate may account for the different sensitivity to the inhibitory effects induced by melatonin [103]. The effect of melatonin seems specific since melatonin precursors, metabolites, or other pineal methoxyindoles, have not been shown to inhibit breast cancer cell proliferation. Melatonin's inhibitory activity is dependent on the pattern (continuous or pulsated) of the exposure to the indole hormone in the culture media; the highest antiproliferative effects are obtained when the concentration of melatonin in culture media is changed every 12 h with concentrations ranging from 10^{-11} to 10^{-9} M, thus mimicking the physiological day/night oscillation of melatonin in the plasma of most mammals [104].

Culture conditions exert a relevant modulation on cell sensitivity to melatonin. In cells growing in anchorage-dependent monolayer culture with FBS, melatonin inhibits MCF-7 cells according to a bell-shaped curve, showing that the highest cytostatic effect is generally obtained around the physiological range (10^{-11}–10^{-9} M). Higher or lower concentrations produce little or no inhibition [68]. Growth-inhibition becomes evident after 48–72 h and thereafter increases linearly up to 144 h [70]. However, in an anchorage-independent culture system, the dose–response curve loses its characteristic form and becomes quite linear with increasing melatonin concentrations producing progressively greater inhibition [105]. This result highlights that cellular attachment to a substratum—that is likely to modify both cytoskeleton and cell shape—plays an important role in setting the level of cell sensitivity to melatonin.

Signaling pathways involved: estrogen pathways

Melatonin influences estrogenic actions on mammary tissue in three different ways: (1) by down-regulating gonadal synthesis of steroids and, consequently, decreasing their circulating levels. Thus melatonin interferes with the systemic effects of estrogens; (2) by interacting with the estrogen-receptor (ER), thus behaving as an anti-estrogen; and (3) by down-regulating the activity of some enzymes, such as aromatase, involved in the synthesis of estrogens from androgens, i.e., behaving as a selective estrogen enzyme modulator [21, 106].

Systemic effects

Melatonin was initially shown to control seasonal reproduction in animals under natural photoperiods [107–109]. In seasonally breeding mammalian species, melatonin controls reproductive function through the

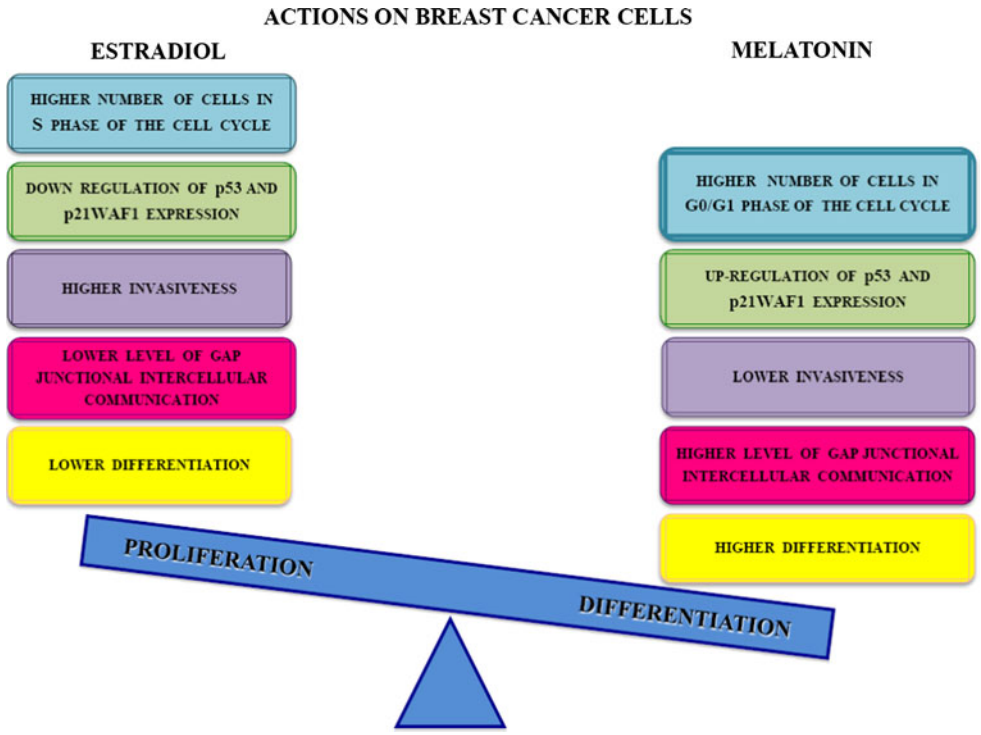

Fig. 1 Melatonin and estrogens have different actions on breast cancer cells. Estradiol stimulates cell proliferation, while reducing differentiation processes; conversely, melatonin promotes differentiation and reduces breast cancer cell proliferation

activation of receptor sites within the hypothalamic-pituitary axis thus driving the levels of gonadal activity [109, 110]. As part of this action, melatonin down-regulates ovarian estrogen secretion in a variety of mammals. It was initially hypothesized that an impaired pineal secretion leads to reduced circulating melatonin levels, which results in unopposed estrogen secretion and thus to an elevated susceptibility to breast cancer [111]. In turn, normal or high serum melatonin levels, by suppressing of estrogen secretion, or by direct inhibitory effects on breast tissue, might restrain induction of mammary cancer (Fig. 1). Although in humans the role of melatonin on the reproductive physiology is not totally clear [112, 113], an inverse relationship between melatonin and ovarian activity [114] and a role of melatonin in the modulation of neuroendocrine-reproductive axis has been proposed [115, 116]. Indeed, melatonin exerts some modulatory actions on steroidogenesis in human granulosa-luteal cells [117]; moreover, functional melatonin receptors have been identified in cells of antral follicles and corpora lutea of rat ovaries [118]. Together, these data suggest that melatonin may participate in the modulation of ovarian function by down-regulating the production of estrogens, thereby supporting the above-mentioned hypothesis of its role in breast cancer.

Melatonin–ER interactions

Only ERα-positive breast tumor cell lines are growth-inhibited by physiologic concentrations of melatonin, whereas ERα-negative cell lines are unaffected by the indoleamine [77, 119]. Various breast cancer cell lines have been reported to exhibit significant differences in their sensitivity to the antiproliferative action of melatonin. This may correlate with the degree of estrogen responsiveness [120] or the ERα/ERβ ratio [67, 121]; indeed, MCF-7 cell sensitivity to melatonin is abolished by ERβ overexpression.

Since melatonin's inhibitory activity has been observed principally in estrogen-responsive breast cancer cells, it has been hypothesized that melatonin hinders cancer cell growth by antagonizing the intracellular estrogen-response pathway. Melatonin blocks the mitogenic effects of estradiol as well as counteracting the estradiol-induced invasiveness of MCF-7 cells [122]. Furthermore, the indoleamine augments the sensitivity of MCF-7 cells to anti-estrogens [123] and down-regulates the expression of proteins, growth factors, and proto-oncogenes regulated by estrogens [124]. Moreover, the transfection of MT1 melatonin receptors into MCF-7 cells or MDA-MB-231 cells (ERα negative) significantly enhances the growth-suppressive effects of melatonin exclusively in MCF-7 cells;

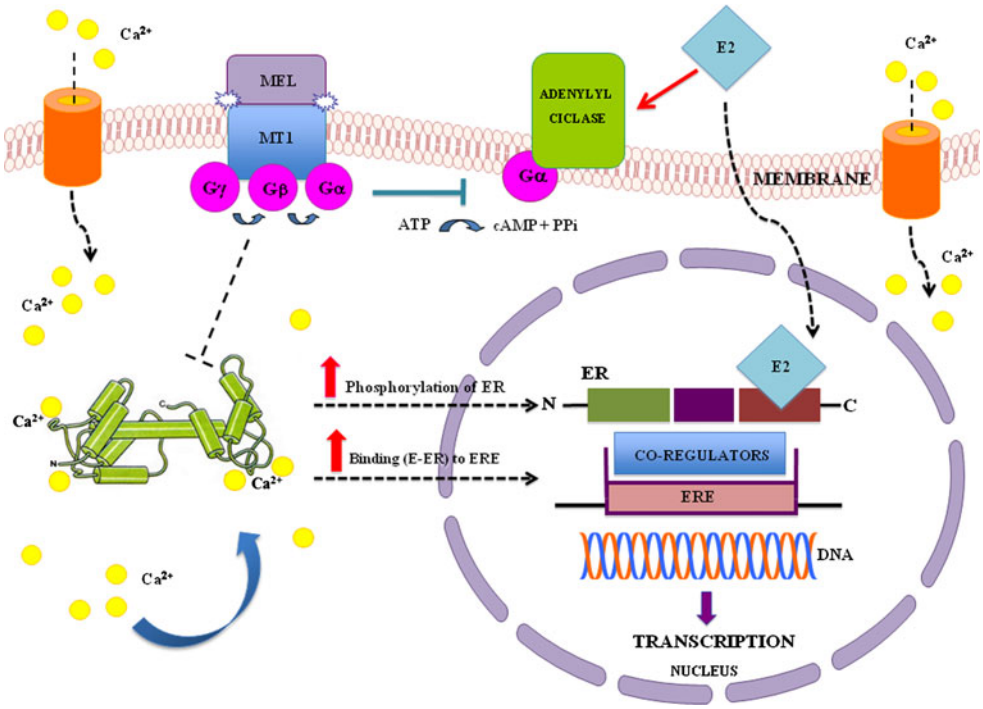

Fig. 2 Melatonin interacts with the Ca^{++}/calmodulin signaling pathway, either by modifying the intracellular accumulation of Ca^{++} or by means of a direct interaction with calmodulin. Calmodulin interacts with ER, stimulating the phosphorylation of the receptor and enhancing the binding of the estradiol–ER complex to ERE (estrogen response elements). Estrogens activate adenylate cyclase and increase cAMP; on the contrary, melatonin, after its binding to MT1, inhibits adenylate cyclase and reduces cAMP

thus, only cells also expressing an estrogen receptor are inhibited [87]. Indeed, melatonin significantly blunts estrogen-induced ERα transcriptional activity, while the addition of pertussis toxin (a known uncoupler of $G_{\alpha i2}$ proteins) suppresses melatonin-induced inhibitory effects [125].

How melatonin interacts with the estrogen pathway remains an open question. Unlike anti-estrogenic drugs, evidence indicates that melatonin does not bind to the ER nor interfere with the binding of estradiol to its receptor [126]. Melatonin reduces the expression of ERα (both at the mRNA and protein level) and inhibits the binding of estradiol–receptor complex to the estrogens response element (ERE) on DNA [47, 127]. These effects likely depend on its binding to a high-affinity membrane-bound receptor coupled to G_i proteins [128] (Fig. 2). Via the activation of $G_{\alpha i2}$ protein, melatonin limits the basal phosphorylation level of the ERα. Thus, melatonin behaves as an anti-estrogen, which does not bind to the ER, but to its own membrane receptors; via this binding to its specific receptors, melatonin interacts with the ER signaling pathway. This effect is specific for ERα-mediated effects. One of the desirable properties of a selective estrogen modulator is its ability to specifically block the ERα but not ERβ. Indeed, it was demonstrated [52] that whereas melatonin is a specific inhibitor of estrogen-induced ERα-mediated transcriptional activation, the indoleamine does not inhibit ERβ-mediated transactivation.

Melatonin and nuclear receptors

The ligand-dependent nuclear transcription factors (NRs) play a multitude of essential roles in development, homeostasis, reproduction, and immune function [129]. NRs regulate transcription by several mechanisms and can both activate and inhibit gene expression [130]. The NRs include steroidal transcription factors such as the estrogen (ER), glucocorticoid (GR), thyroid hormone receptor (TR), liver X receptor (LXR), farnesoid X receptor (FXR), vitamin D receptor (VDR), retinoid acid receptor (RAR), retinoid X receptor (RXR), and peroxisome proliferators-activated receptors (PPARs) [131]. Through its role as a required heterodimeric partner, RXRs control the function of many other NRs, thus integrating a unique transcriptional network dependent on RXR responses [132, 133]. RXR forms heterodimers with virtually all NRs including GR, ER, TR, PPAR, VDR, LXR, and FXR. NRs can activate transcription as monomers and/or dimers with the RXR. Once activated, NRs dissociate from co-repressors and recruit co-activator proteins, which promote transcriptional activation [134, 135]. Half of the NRs are so-called "orphan" receptors because the identity of their

ligand is unknown. However, some receptors belonging to RXRs are no longer "orphaned". Evidence of a genomic action of melatonin via nuclear RZR/ROR receptors was initially hypothesized by Becker-Andre in 1994 [136]. Subsequent studies have detected the nuclear melatonin receptor by using in situ hybridization in neuronal tissue [137, 138] including the pineal gland [139] and in non-neuronal other tissues as well [140–142].

Direct evidence of the epigenetic effect of melatonin has been provided by Sharma et al. [143]. In this study, melatonin significantly elevated mRNA expression for various histone deacetylases (HDAC) isoforms and increased histone H3 acetylation in neural stem cell lines. As suggested by Korkmaz and colleagues [144, 145], these effects are indicative of an epigenetic regulation exerted by melatonin at NR/co-regulator level rather than selective enzymatic inhibition or activation. It is not still known if melatonin's effects are mediated via direct changes in phosphorylation of the nuclear receptor, regulation of coactivator or corepressor phosphorylation, or both [146]. Regardless, these data clearly demonstrate the ability of melatonin, via signal transduction pathways, to influence gene expression in human breast cancer cells.

Melatonin as calmodulin and calcium modulator

Even if the molecular link between melatonin and estrogen pathways has not been fully elucidated, it is likely that cyclic AMP (cAMP) could, at least in part, modulate this function. Estrogens activate adenylate cyclase, thereafter increasing c-AMP levels; in turn, c-AMP synergizes with hormone receptors, enhancing ER-mediated transcription [147]. On the contrary, melatonin, after it binds to MT1, it inhibits adenylate cyclase, reduces c-AMP and in turn protein kinase A (PKA) activity leading to a diminished phosphorylation and activation of ERα and the co-activators CBP/p300, thus blocking the estrogenic effect [148, 149].

It has recently been proposed that melatonin may hinder the estrogen pathway through the Ca^{++}/calmodulin signaling pathway, either modifying the intracellular accumulation of Ca^{++} or by means of a direct interaction with calmodulin (CaM) [150, 151]. Furthermore, melatonin changes CaM subcellular redistribution stimulating its phosphorylation by protein kinase C (PKCα) [152]. It is known that calmodulin interacts with ER, stimulating the phosphorylation of the receptor and thus enhancing the binding of the estradiol–ER complex to the ERE [153]. Conversely, anti-calmodulin compounds inhibit breast cancer growth, probably by interfering with the CaM-ER interplay [154]. Since both melatonin and calmodulin are phylogenetically well preserved, calmodulin–melatonin interaction probably represents a major mechanism for regulation and synchronization of cell physiology and it is likely that melatonin interference with calmodulin functions could contribute to modulate estrogen receptor activation. Moreover, the melatonin-induced rise in both intracellular Ca^{++} and membrane-bound calmodulin could enhance apoptosis and E-cadherin mediated cell–cell adhesion [155]. Consistent with this, Blask et al. [156] reported that melatonin inhibits Ca^{++}-stimulated MCF-7 cell growth via a glutathione-dependent mechanism. Indeed, once glutathione synthesis is inhibited using buthionine sulfoximine (an inhibiter of γ-glutamylcysteine synthetase) the oncostatic action of 1 nM melatonin was blocked, indicating that glutathione is required for melatonin action [157]. In addition, glutathione depletion has been shown to cause a reduction in microtubule polymerization in cells that may relate to the oxidation of sulfhydryl groups [158]. In contrast, physiological concentrations of melatonin are known to stabilize microtubules by inhibiting Ca^{++}/CaM depolymerization, which is itself a mitogenic signal transduction mechanism [159]. Thus, adequate levels of glutathione may be required to maintain the sulfhydryl groups of microtubule-associated proteins in a reduced state in order for melatonin to suppress Ca^{++}/CaM-mediated depolymerization of the cytoskeleton and thus cell proliferation. These effects are seemingly to be ER-independent, keeping in mind that they have been recorded also in ER-negative breast cancer cells, as well as in non-breast cancer cell lines devoid of the ER; these data therefore could provide a reasonable hypothesis about how melatonin inhibits cell proliferation [44, 160, 161] (Fig. 2). Clearly, further studies are warranted in order to verify and better understand the physiological meaning of melatonin's modulation of calmodulin and intracellular calcium in breast cancer cells.

Aromatase pathways

In MCF-7 breast cancer cells, as well as in adipose tissue of tumor-bearing breasts, expression of the CYP19 gene, which encodes aromatase P450, the enzyme responsible for estrogen biosynthesis, is regulated by two proximal promoters, i.e., I.3 and II [162], mainly modulated by intracellular cAMP [163]. Therefore, molecules or drugs able to modulate cAMP levels could also influence aromatase expression in breast cancer cells. This is the case with prostaglandin E2 (PGE_2) that increases intracellular cAMP levels and stimulates aromatase and estrogen biosynthesis [164]. Estrogens also increase cAMP, as previously mentioned. Thus, in breast cancer cells, but not in normal epithelial cells with different CYP19 promoters, estrogens may induce, through a paracrine loop, the local biosynthesis of estrogens via the increase of cAMP and expression of aromatase. On the other hand, melatonin, after its binding to MT1 membrane receptor linked to G_i proteins, decreases in a dose- and time-dependent manner

the activity of adenylate cyclase and subsequently reduces cAMP synthesis thus leading to elevated cGMP levels and a reduced aromatase concentration [165]. Indeed, it has been observed that melatonin, at both physiological (10^{-9} M) and pharmacological (10^{-4} M) concentrations, reduces the synthesis of estrogen in MCF-7 cells, through aromatase inhibition [166]. Furthermore, transfection of the MT1 melatonin receptor in MCF-7 cells significantly reduced aromatase activity and MT1-transfected cells showed a level of aromatase activity that was 50 % of that of the control cancer cells when both are treated with melatonin [167]. Moreover, melatonin enhances the inhibitory effect of aminoglutethimide on aromatase activity in breast cancer cells, through a significant reduction in aromatase mRNA expression [168]. Additionally in MCF-7 cells, aromatase activity is stimulated by epidermal growth factor and transforming growth factor-α [169], both of which are down-regulated by melatonin [170]; melatonin-dependent aromatase inhibition could be further achieved through suppression of cyclooxygenase activity and reduced prostaglandin E2 synthesis [171].

As breast cancer occurs in regions of the mammary gland with the highest levels of aromatase expression, the inhibition of aromatase activity by melatonin may be an important mechanism in the ability of this indoleamine to control tumor growth. Other studies confirmed that melatonin efficiently inhibits local, tissue-based biosynthesis of estrogen. It is well recognized that mammary cancer tissue contains all the enzymatic machinery for the local biosynthesis of estrogens [172]. The presence of the type 1 (17β-HSD1) isoform of 17β-hydroxysteroid dehydrogenases, which catalyze the conversion of the relatively weak estrone (E1), androstenedione and 5-androstenedione to the more potent estradiol, has been documented in several human breast cancer cell lines, including T47D and MCF-7 [172]. Estrogen production in normal mammary tissue is displaced toward the production of hormones with low activity (like estrone), whereas in breast adenocarcinomas what predominates is the formation of the active estradiol [173]. This effect is likely to be due to the different enzyme composition of normal and cancerous tissues. The former exhibits a higher activity of both 17β-HSD type 2, which converts estradiol to estrone, and estrogen sulfotransferase, which inactivates both estrone and estradiol; on the other hand, opposite effects are mediated by aromatase and type 2 isoform of 17β-hydroxysteroid dehydrogenase that are largely represented in cancer tissues. It has been demonstrated that melatonin reduces the synthesis of biologically active estrogens in MCF-7 cells, through the contemporary inhibition of sulfatases and 17β-HSD1 and the stimulation of estrogen sulfotransferase, the enzyme responsible for the formation of the biologically inactive estrogen sulphates. As a result, the production of estradiol from estrone in MCF-7 cells decreases two- to three-fold in the presence of melatonin 1 nM [174].

The transcription factor nuclear factor κB (NF-κB) is involved in CYP19 activation by inducing several pro-inflammatory molecules [tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2), and PGE_2] [175]. Over-expression of TNF-α, COX-2, and PGE_2 has been demonstrated to induce elevated aromatase expression in both human and mice breast cancer tissue [72, 176]. It is noteworthy that melatonin inhibits every molecule in this pathway, mainly through its nuclear actions [177, 178]. Moreover, melatonin inhibits p300 HAT activity, thus leading to a reduced COX-2 and iNOS synthase expression [179]. This effect is likely to be mediated by inhibition of p52 acetylation and binding of DNA. As expected, melatonin also suppresses NF-κB binding to DNA [180–182], thereby decreasing TNF-α, iNOS, COX-2 and PGE_2 levels. Furthermore, melatonin interaction with PPARs and RXR hinders NF-κB transcription, leading to cancer cell growth inhibition [183, 184]. In turn, melatonin-induced activation of both PPARs and RXR receptor inhibits aromatase transcription via NF-κB. Melatonin also inhibits the expression of other estrogen-regulated genes, like pS2 or cathepsin [185]. These findings are important, as pS2 has been demonstrated to be a differentiation factor in the gastrointestinal tract, as well as an inhibitor of adenocarcinoma cell proliferation [186, 187].

Growth-inhibitory mechanisms

Melatonin significantly limits cancer proliferation in vitro. For melatonin to achieve this effect, it seems several mechanisms are involved, including actions on the expression of some proteins involved in the control of the G_1–S transition, through the inhibition of cyclin D1 expression and the increase in p53 release (Fig. 3). Cyclin D1 is a key protein of G_1 to S transition and seems to mediate the steroid-dependent growth of both normal and malignant mammary epithelial cells [188]; moreover, down-regulation of cyclin D1 expression may be sufficient to drive the inhibitory effects displayed by anti-estrogenic drugs [189]. Cyclin D1 interacts with several transcription factors as well as with nuclear receptors (including GR, ERα, and PPARs). NRs directly bind to cyclin D1 and their ligand-dependent transactivation is modulated by cyclin D1 [190]. It is noteworthy that cyclin D1 participates in the activation process of ERα transcription and cooperates in the down-regulation of both GR and PPARs [191]. Melatonin induces a significant transcriptional down-regulation of the cyclin D_1 gene through the inhibition of c-*jun* and ATF-2 proteins [192]. Both c-*jun* and ATF-2 proteins are known to transactivate the cAMP-responsive element present in the cyclin D_1 promoter element [193].

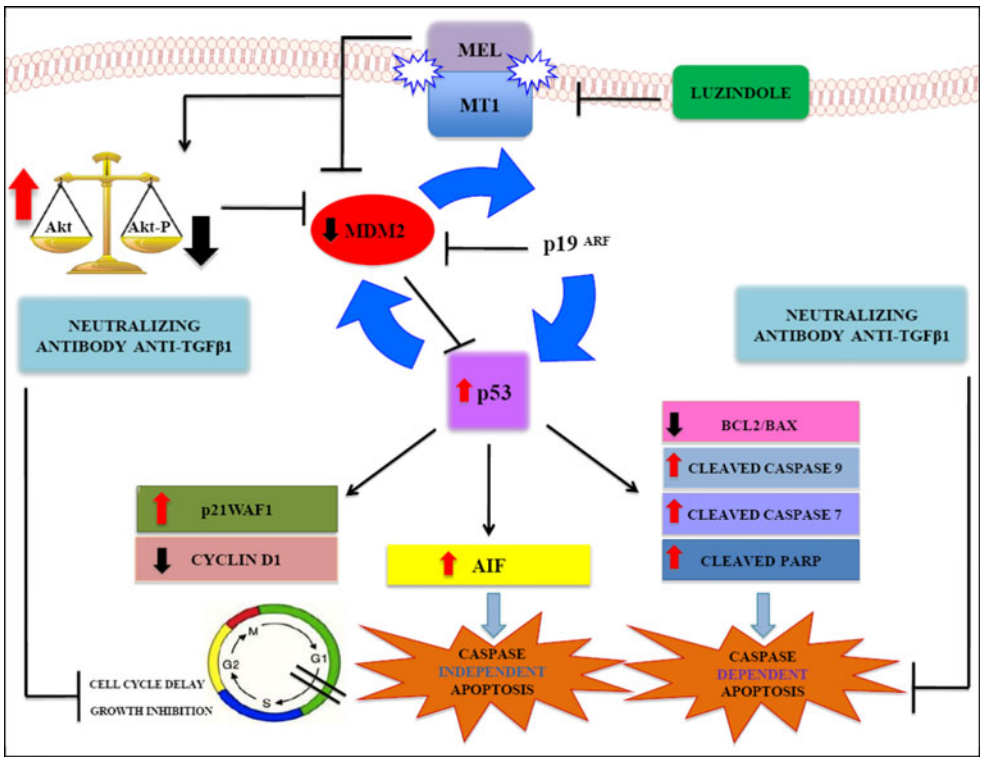

Fig. 3 Melatonin significantly inhibits cancer proliferation by increasing the p53/MDM2 and Akt/Akt-P ratios. The p53 gene activates the expression of p21, which inhibits cyclin-dependent kinases, thus leading to cell cycle arrest. In addition, melatonin induces apoptosis in MCF-7 cancer cells. Melatonin-mediated early apoptosis is a caspase-independent process, involving the apoptosis-inducing factor (AIF). Melatonin-induced late apoptosis is TGFβ-1 and caspase-dependent process. During late apoptosis, activated caspase-9, -7, and cleaved-PARP increase significantly, concomitant with a down-regulation of the Bcl/Bax ratio. By adding anti-TGFβ-1 neutralizing antibodies, growth inhibition and late apoptosis triggered by melatonin are inhibited

The p53 gene is involved in both growth suppression and apoptosis pathways [193]. The p53 gene activates the expression of the WAF1 gene (also known as p21), which inhibits cyclin-dependent kinases thus leading to a failure of the phosphorylation of the retinoblastoma protein and the subsequent cell cycle arrest [194].

In breast cancer cells treated with physiological doses of melatonin, both p53 and p21 expression is significantly augmented [195]. It is likely that up-regulation of p53 occurs downstream to enhanced release of TGFβ-1 induced by melatonin. Indeed, melatonin can up-regulate TGFβ-1 mRNA expression in prostate [196] and breast cancer cells [197]. Moreover, melatonin-inhibitory effect on breast cancer growth should be viewed as a TGFβ-1 dependent process as it can be completely prevented in several breast cancer cell lines by adding anti-TGFβ-1 antibodies [70, 74, 198]. A significant rise in TGFβ-1 levels in MCF-7 cells treated with melatonin are measured after 72 h and an evident growth-inhibitory action is documented only after this period. After adding anti-TGFβ-1 antibodies, the growth-inhibition induced by melatonin was completely prevented. These data point out that melatonin-induced cell-growth inhibition in MCF-7 breast cancer cells is largely mediated through the involvement of the TGFβ-1 pathway.

Other mechanisms are also involved in melatonin-mediated inhibition of cancer cells. The decrease in cAMP production caused by melatonin via MT1 and MT2 receptor interaction reportedly slows down the uptake of linoleic acid, an essential fatty acid, by specific fatty acid transporters [199]. Linoleic acid can be oxidized to 13-hydroxyoctadecadienoic acid by 15-lipoxygenase, serving as an energy source for tumor growth and tumor growth signaling molecules. Inhibition of linoleic acid uptake by melatonin is regarded as one mechanism of its antiproliferative effects.

Melatonin hinders telomerase activity, induced by estrogens or cadmium, both in vitro and in vivo [200, 201]. Melatonin-treated cells display a significant dose-dependent decrement in telomerase reverse transcriptase mRNA expression as well as the mRNA of telomerase-reverse, the RNA telomerase subunit. Similar results have been obtained with GP 52608—an agonist of melatonin nuclear receptors—while treatment with an agonist of melatonin membrane receptors did not produce any effect, thus

highlighting the relevance of epigenetic mechanisms triggered by melatonin [202]. Telomerase is a specialized ribonucleoprotein DNA polymerase that extends telomeres of eukaryotic chromosomes and its activity is under control of epigenetic regulation [203]. Telomerase is activated in most human cancers and its inhibition leads to cancer cell death. Therefore, it is tempting to speculate that such a mechanism could also be involved in melatonin-dependent cancer apoptosis [204].

Apoptosis pathways

In contrast to the well-studied inhibition of apoptotic processes by melatonin in normal cells [205, 206], it is well known that in cancer cells, melatonin actually promotes apoptosis. Melatonin induces programmed cell death in colon cancer cells [207–209], hepatocarcinoma cells [201, 210], neuroblastoma [211], Ehrlich ascites carcinoma cells [212], myeloid [213], lymphoma [214], pancreatic cancer [215, 216], renal cancer [217], and leukemia cells [218–220]. However, although inhibitory effects of melatonin in MCF-7 have been well documented, the mechanism by which the apoptotic effects are executed are still a matter of investigation. Cos and coworkers [221] claimed no apoptotic effects on MCF-7 cells treated with different concentrations of melatonin, while other reports [222, 223] documented a significant rise in MCF-7 cells apoptotic rate when melatonin was administered together with retinoids. In the latter studies, melatonin was able to enhance apoptosis by modulating the transcriptional activity of the retinoic acid receptors [224]. A recent study documented a significant increase in caspase-3 activity and DNA fragmentation in tumor tissues obtained from breast cancer-bearing rats treated with melatonin, therefore providing at least an indirect proof of the apoptotic activity exerted by melatonin on breast cancer [225].

These apoptotic actions of melatonin have been confirmed in vitro, where treating MCF-7 cells with nanomolar concentrations of melatonin caused cell death [70]. Both flow cytometry and DNA fragmentation-based techniques documented an early (at 24 h) and a late (at 96 h) apoptosis in melatonin-treated MCF-7 cells. Early apoptosis is a caspase-independent process, and it is likely to be triggered by the apoptosis-inducing factor (AIF). In contrast, a more complex pathway underlies late apoptosis, involving both TGFβ-1 and terminal caspase effectors (caspases-7). Indeed, adding anti-TGFβ-1 antibodies, melatonin-induced late-apoptosis is almost completely suppressed, while early apoptosis remains unaffected. During late apoptosis, activated caspase-9 and -7 and cleaved-PARP increased significantly, concomitantly with a down-regulation of the Bcl-2/Bax ratio. It is noteworthy that melatonin-triggered apoptosis involves both p53 and p73 release. In fact, melatonin-treated MCF-7 cells showed a significant rise in both p73 and p53, but only the p73 protein, the homologue of p53 protein, increased at 96 h; concurrently, MDM2 levels were significantly reduced. These data suggest that p53 is likely activated during early programmed cell death, while only p73 is involved in caspase-dependent late-apoptosis.

It is worth reiterating that MDM2 is decreased as a consequence of melatonin treatment. MDM2 inhibits the transcriptional activity of p53 and promotes its degradation by the proteasome, thus representing the major physiological antagonist of p53 [226]. An autoregulatory negative feedback loop controls the MDM2 expression, where p53 induces MDM2 expression, whereas MDM2 represses p53 activity. Abrogation of the MDM2 expression allows the p53 to escape from the autoregulatory loop, becoming lethally active [227, 228]. Thus, melatonin induces an early down-regulation of MDM2 expression concomitant with a p53 rise, causing a significant rise in the p53/MDM2 ratio (Fig. 4). It is likely that the modified p53/MDM2 ratio could trigger the apoptotic cascade involving both caspase-dependent and caspase-independent pathways.

We have recently found (unpublished results) that melatonin enhances the depolarization of the mitochondrial membrane, while inhibiting Akt-phosphorylation; these effects are probably involved in melatonin-dependent oncostatic effects and they participate in triggering the complex apoptotic cascade. Similar results have been obtained by adding melatonin together with vitamin D3 [229]. Melatonin and vitamin D3 induced in MCF-7 cells a synergistic proliferative inhibition, with an almost complete cell growth arrest at 144 h. Cell growth blockade is associated to an activation of the TGFβ-1 pathway, leading to increased TGFβ-1, Smad4, and phosphorylated-Smad3 levels. Concomitantly, melatonin and D3, alone or in combination, caused a significant reduction in Akt phosphorylation and MDM2 values, with a consequent initial elevation of p53/MDM2 ratio [229]. These effects were completely suppressed by adding a monoclonal anti-TGFβ-1 antibody to the culture medium.

As Sainz and colleagues [205] reported, "melatonin involvement in apoptotic processes is a new and relevant field of investigation. The results obtained to date appear promising, and if in fact melatonin uniformly induces apoptosis in cancer cells, the findings could have important clinical utility. Many tumors show resistance to drug treatment mainly due to their resistance to undergo apoptosis. Identifying agents which potentiate apoptosis in cancer cells is clearly of great interest". It may seem paradoxical that a substance could induce apoptosis in cancer cells, while preventing this process in normal cells. However, melatonin shares this unusual behavior with other known anti-oxidant compounds, including epigallocatechins and procyanidins [230]. Clearly melatonin's ability to

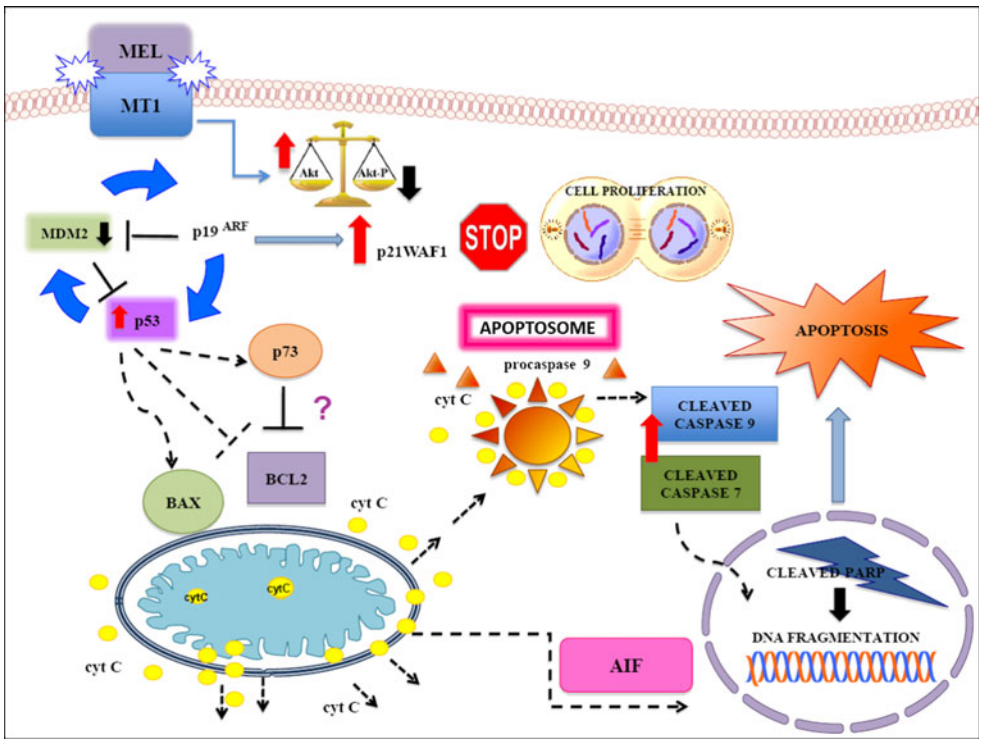

Fig. 4 After binding to the MT1 receptor, melatonin reduces MDM2 levels, thus allowing p53 to escape from the autoregulatory loop. The modified p53/MDM2 ratio triggers the apoptosis cascade involving both caspases-dependent and caspases-independent pathways. In addition, melatonin increases mitochondrial membrane depolarization, releasing cytochrome C and apoptosis inducing factor (AIF). Melatonin is likely to inhibit AKT phosphorylation and subsequently the MAPK-related pathways

trigger apoptotic or anti-apoptotic pathways is largely context-dependent.

Malignant behavior

Some preliminary observations suggest that melatonin can efficiently reduce the metastatic ability of MCF-7 cells. Mao et al. [231] have evaluated the potential anti-invasive actions of melatonin, employing three clones of MCF-7 cells with high metastatic potential including the MCF-7/6 clone derived by serial passages in nude mice, MCF-7Her2.1 cells stably transformed with and overexpressing the Her2-neu/c-erbB2 construct, and MCF-7CXCR4 cells stably transformed with and overexpressing the CXCR4 cytokine G protein-coupled receptor. The invasive capacity of these clones was significantly reduced when they were treated with melatonin (10^{-8} or 10^{-9} M). Melatonin treatment resulted in marked suppression (60 to 85 % decrease) of cell invasion using a Transwell assay system and Matrigel-covered inserts.

In an in vitro study, Cos et al. [232] demonstrated that 1 nM melatonin reduced the invasiveness of cancer cells measured in Falcon invasion chambers and also blocked estradiol-induced invasion [123]; both sub-physiological (0.1 pmol) and pharmacological concentrations (10 μM) of melatonin failed to inhibit cell invasion. It is likely that such effects could be attributed to an overall effect of melatonin on cell morphology, which are mediated, at least in part, by a melatonin-induced elevation in the expression of two cell-surface adhesion proteins, E-cadherin and β1-integrin, as well as by an increased gap junctional intercellular communication between adjacent epithelial cells also induced by melatonin [232]. It has been hypothesized that if a cell would be unable to perform gap junctional intercellular communication, normal growth control and cell differentiation would not be possible, thereby favoring the development of malignant neoplasia [233]. Since there is an inverse correlation between the ability of a cell to exhibit gap junctional communication and its ability to metastasize [234], it is likely that melatonin may reduce metastatic behavior through inducing local gap junctional intercellular communication and re-shaping the relationships between cancer cells and their microenvironment.

Estrogen treatment is known to induce a marked rearrangement of the cytoskeleton and adhesion structures and enhances the attachment of MCF-7 cells to laminin (a basement membrane component); this action is completely abolished by melatonin and shifts cancer cells to a lower invasive status [235]. These findings indicate that melatonin

could exert an additional anti-tumor action by modulating the cross-talk between cells and stroma components (stromal cells, laminin, collagen), and by increasing regulatory signals shared between adjacent cells through intercellular junctions. Also, cancer cell invasiveness is enhanced by the elevated activities of matrix metallo-proteinases which break down interstitial tissue thereby aiding cancer cell movement and access to blood vessels. Since melatonin inhibits the activities of the proteinases that cause the dissolution of the connective tissue elements [236], the indole may also impede cancer cell invasiveness by this means.

Melatonin, cytoskeleton, and cell morphology

From the initial studies carried out by Hill et al. [67], it was already apparent that melatonin significantly modifies breast cancer cell morphology and cytoskeleton architecture. Likewise, melatonin also changes the histomorphology of prostate cancer cells and renders them more sensitive to cytokine-mediated apoptosis [237, 238]. The significance of these findings has been underestimated since only recently compelling evidence has been provided documenting the relevance of melatonin's influence on the cytoskeleton.

The cytoskeleton is an important group of cellular structures, composed of an intricate fibrous network including microtubules, microfilaments, and intermediate filaments as well as their associated proteins [239]. Dynamic and differential changes in cytoskeletal organization occur during different cellular processes according to the cell type and the specific function. Moreover, the cytoskeleton, together with integrins and other related adhesion proteins, orients much of the metabolic and signal transduction machinery of the cell [240]. Indeed, cells are hard-wired to respond immediately to mechanical stresses transmitted over cell surface receptors that physically couple the cytoskeleton to the extracellular matrix or to other cells [241]. The shape of cells and the internal structure are consequences of physical forces generated in the cytoskeleton as well as in extracellular matrix. Shape, in reflecting cytoskeleton organization [242], is linked to a repertoire of metabolic events, which result from the right ordering in space of the enzymes catalyzing specific pathways. Physical forces (e.g., microgravity) induce dramatic changes in gene expression and alter cellular shape [243, 244]. In particular, distortions in cell shape can switch between distinct cell phenotypes, and this process is viewed as a biological phase transition [245, 246]. Moreover, tumor phenotype reversion is primarily associated with relevant shape modifications, preceding molecular and metabolic "normalization" [247].

The cytoskeleton is a phylogenetically well-preserved structure allowing the cell to have a well-organized structure, a specific shape-associated phenotype, and an optimal functionality. By contrast, cancer is characterized by an abnormal cytoskeletal rearrangement with poor organization and structure [248]. Therefore, the ability of melatonin to preserve normal microfilament distribution and its influence on cytoskeleton rearrangement and cell shape in cancerous tissues deserves further investigation [249].

Cancer cells show an abnormal microfilament organization, reduced stress fiber production, and loosened focal contact adhesion. These changes enhance cell proliferation, cell migration [250], and foster resistance to apoptosis [251]. Highly malignant metastatic cancer cells present poorly structured microfilaments and scarce anchorage to their substratum. These cells have microfilaments and microtubules arranged in membrane ruffles, lamellipodia, and filopodial formations at the leading edge; at the cell rear, a retraction of these cytoskeletal structures occurs [252].

A bimodal effect of melatonin on microtubule organization was first described in 1994 in both in vitro polymerization assays and in a preparation of cytoskeleton material in situ [253]. Melatonin, in the presence of Ca^{++}, augmented tubulin polymerization, causing microtubule enlargement, while melatonin without Ca^{++} inhibited tubulin polymerization and caused microtubule disruption. Interestingly, in kidney epithelial cells (MDCK), melatonin's effects on cytoskeletal organization are not mediated by membrane melatonin receptors, while in breast cancer cells, luzindole, an MT1 and MT2 antagonist, completely prevented melatonin-induced effects on microfilaments. Furthermore, studies performed by Benitez-King et al. [254] have demonstrated the complex interaction between melatonin and the cytoskeleton. In experimental conditions designed to measure cell anchorage, melatonin increases the number of focal adhesion contacts by MCF-7 cells, and microfilaments are arranged in thicker bundles of stress fibers assembled with phospho-vinculin to form focal adhesion contacts [255]. These results strongly suggest that melatonin inhibits cancer cell invasion and metastasis formation by changing microfilament phenotypes of migratory cells (ruffles and lamellipodia) to stress fibers that are microfilament phenotypes of attached cells.

Melatonin-induced effects on stress fibers involve protein kinase C (PKC) and the Rho-associated protein kinase (ROCK), downstream of the PKC pathway [256]. In MDCK and MCF-7 cells treated with melatonin, the addition of the PKC inhibitor abolished the augmented number and thickening of stress fibers, as well as the elevated number of focal adhesion contacts elicited by the indoleamine in both cell lines [257]. It is noteworthy that calmodulin also participates in this process [258] and that melatonin modulates stress fiber formation by involving ROCK and Ca^{++}/CaM balance. In MCF-7 cells, melatonin

causes a redistribution of CaM and phosphorylated-CaM, recruiting them to specific subcellular compartments, one of which is the cytoskeleton [259]. CaM is redistributed to the membrane cytoskeletal fraction where it becomes associated to myosin phosphorylation, through the myosin light-chain kinase [159].

Conclusions

Numerous studies have documented the oncostatic properties of melatonin both in vivo, with models of chemically induced rat mammary tumors, as well as in vitro using MCF-7 human breast cancer cells. Melatonin exerts both inhibitory as well pro-apoptotic effects, interacting with several molecular pathways. Generally, melatonin's cytostatic actions seem to be mediated by the interaction of the indoleamine with both the estrogen receptors and the melatonin receptors. However, recently, some receptor-independent and estrogen-independent signaling pathways activated by melatonin have been uncovered. In particular, increasing attention should perhaps be directed to melatonin's effects on the cytoskeleton and cell shape, as well as identifying how melatonin inhibits both Akt activation and MAPK-related pathways. A recent paper illustrates how melatonin exerts an inhibitory effect on breast cancer cell invasion through down-regulation of the p38 pathway and inhibition of MMP-2 and MMP-9 expression and activity [236]. The anticancer effects triggered by melatonin could be at least in part mediated by a selective genetic modulation, as it was shown that a set of microRNAs are differentially up- or down-regulated by melatonin in MCF-7 cells [260].

Additional research is required to clarify if melatonin administration constitutes, either alone or in combination with chemo-radiotherapy [261], a potentially new anticancer treatment. Given its widespread actions on breast cancer, its virtual absence of toxicity and its low cost, it seems reasonable to strongly recommend more thorough trials as to its usefulness as a preventive or treatment of breast cancer.

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