Breast Cancer Research and Treatment (2005) 94: 249–254
DOI 10.1007/s10549-005-9006-x
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Report

Melatonin enhances the inhibitory effect of aminoglutethimide on aromatase activity in MCF-7 human breast cancer cells

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Key words: aromatase, breast cancer, MCF-7, melatonin, pineal

Summary

The inhibition of the aromatase-induced intratumoral estrogen synthesis is one of the main anticancer pharmacological strategies. The aim of this paper was to study if a melatonin pretreatment prior to aminoglutethimide increases the efficiency of the aromatase inhibitor used in treating breast cancer. Aminoglutethimide (100 μM) and melatonin (1 nM) significantly decreased cellular aromatase activity in unpretreated MCF−7 cells. A sequential regimen of melatonin (1 nM) followed 24 h later by aminoglutethimide (100 μM) induced a significantly higher decrease in MCF-7 cell aromatase activity to below the values obtained in unpretreated cells. Melatonin treatment inhibited aromatase mRNA expression in unpretreated cells and a sequential treatment of cells with melatonin followed by aminoglutethimide induced a significant inhibition in the aromatase mRNA expression as compared to cells exposed to the same doses of aminoglutethimide, but without melatonin pretreatment.

The present study demonstrates that a treatment with melatonin followed by aminoglutethimide is the most effective way of reducing the aromatase activity in the MCF-7 cell line. The aminoglutethimide inhibitory effect is more potent when MCF-7 cells are pre-exposed to melatonin. Our results suggest that melatonin pretreatment increases the reduction of the aromatase activity of cells exposed to aminoglutethimide as a result of the decrease in the aromatase mRNA expression. The findings presented here point to melatonin pretreatment as a novel and interesting means to increase the efficacy of competitive aromatase inhibitors used in treating breast cancer.

Introduction

Estrogens play a significant role in the development and growth of breast cancer. In the hormone-dependent subtype, the role of estrogens as modulators of tumor growth overrides the influence of other factors. The tumorigenic effect of estrogen is manifested, in part, through promotion of breast epithelial cell growth [1]. Based upon the concept that estrogen is the proximate regulator of cell proliferation, two different pharmacological strategies have been employed to selectively neutralize the effects of estrogens on mammary cells: (a) development of drugs that act through the estrogen receptor interfering with the effects of the endogenous estrogens; this group includes the selective estrogen receptor modulators (SERMs), of which tamoxifen and its derivatives are the most representative examples; (b) development of drugs that interfere with the synthesis of steroid hormones by inhibiting the enzymes controlling the interconversion from androgenic precursors, the so-called selective estrogen enzyme modulators (SEEMs) [1–3].

Melatonin, the main pineal hormone, has been shown to inhibit both cancer initiation and cancer cell growth, particularly on endocrine-responsive breast cancer [see reviews 4–7]. Different hypotheses, including the melatonin immunomodulatory actions [8], its antioxidative effects [9], or inhibition of telomerase activity [10] have been postulated to explain the oncostatic properties of melatonin; however, the effects of this indoleamine on mammary cancer have been largely considered as a consequence of its antiestrogenic actions [11]. At this moment, melatonin fulfils all the requirements to be considered as an antiestrogenic drug and we can state that this indolamine could influence estrogen-mediated cancer growth in three different ways: (a) by down-regulating gonadal synthesis of steroids and, consequently, decreasing their circulating levels; (b) by interacting with the estrogen receptor, decreasing the expression of estrogen receptor and inhibiting the binding of the estradiol-estrogen receptor complex to the estrogen response element on the DNA, thus behaving as a selective estrogen receptor modulator; and (c) by down-regulating the activity of some enzymes, such as aromatase, involved in the synthesis of estrogens from androgens, that is, behaving as a selective estrogen enzyme modulator [11].

Although antiestrogen therapy with tamoxifen has been the preferred treatment of estrogen responsive

breast cancer, there is currently a shift towards treatment with aromatase inhibitors due to a growing body of evidence supporting the role of aromatase inhibitors [12,13]. Aminoglutethimide, the first non-steroidal aromatase inhibitor used clinically in mammary cancer, is a reversible competitive inhibitor which binds competitively to the heme moiety of aromatase. Melatonin is a hormone that, at physiological concentrations, besides having antiestrogenic actions, reduces the aromatase activity and downregulates aromatase mRNA steady state levels in MCF-7 cells [11]. Aromatase inhibitors and antiestrogens have shown substantial activity in primary and advanced breast cancer. Since they exhibit different modes of action, attempts have been made to combine them or to use them sequentially in order to potentially increase their efficacy [14]. Therefore, the aim of this paper was to test the hypothesis that a sequential or alternating treatment with melatonin and aminoglutethimide increases the efficacy of the aromatase inhibitor. The question under study was whether a short-term melatonin pretreatment increases the sensibility of MCF-7 cells to the subsequent inhibitory effect of either aminoglutethimide or melatonin itself.

Materials and methods

Cells and culture conditions

MCF-7 human breast cancer cells were purchased from the American Tissue Culture Collection (Rockville, MD, USA). They were maintained as monolayer cultures in 75 cm^2 plastic culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Chemical Co., St Louis, MO, USA) supplemented with 5% fetal bovine serum (FBS) (Gibco, France), penicillin (20 units/ml) and streptomycin (20 μg/ml) (Sigma Chemical Co., St. Louis, MO, USA), at 37 °C in a humid atmosphere containing 5% CO_2. Cells were subcultured every 3–4 days by suspension in 5 mM Na_2-EDTA in PBS (pH 7.4) at 37 °C for 5 min.

Before each experiment, stock subconfluent monolayers (80%) of MCF-7 cells were incubated with 5 mM Na_2-EDTA in PBS (pH 7.4) at 37 °C for 5 min, resuspended in DMEM supplemented with 5% FBS and passed repeatedly through a 25-gauge needle to produce a single cell suspension. Cell number and viability were determined by staining a small volume of cell suspension with 0.4% trypan blue saline solution and examining the cells in a hemocytometer.

Measurement of cellular aromatase activity

Aromatase activity in MCF-7 cells was measured by the tritiated water release assay, based on the formation of tritiated water during aromatization of a labeled androgenic substrate such us [1β-^{3}H(N)]-androst-4-ene-3,17-dione [15]. MCF-7 cells were cultured in 75 cm^2 plastic culture flasks in DMEM supplemented with 10% FBS, 20 units/ml penicillin and 20 μg/ml streptomycin, at 37 °C in a humid atmosphere containing 5% CO_2. After 48 h, media were aspirated and replaced by fresh media with and without 1 nM melatonin (Sigma-Aldrich Química S.A., Madrid, Spain). MCF−7 cells were incubated in either melatonin-containing or control culture media for 24 h. After this, both those cells cultured with and without melatonin were harvested and seeded onto 60 × 15 mm tissue culture dishes (1.5 × 10^6 cells/dish) in DMEM supplemented with 5% charcoal-stripped FBS (sFBS) and incubated for 48 h. Then, the medium was changed and replenished with fresh ones (1 ml per plate) containing 100 nM [1β-^{3}H(N)]-androst-4-ene-3,17-dione] (NEN Life Science Products, Boston, MA, USA) (25–30 Ci/mM) in the presence of either 1 nM melatonin, 100 μM of the aromatase inhibitor aminoglutethimide (Sigma-Aldrich Química S.A., Madrid, Spain), 1 nM melatonin plus 100 μM aminoglutethimide, or the diluent of these drugs (ethanol at a final concentration lower than 0.0001%) (Figure 1). After 24 h of incubation, all culture dishes were placed on ice for 15 min to condense any water vapor and the media were transferred to tubes containing 0.25 ml ice-cold 30% trichloroacetic acid (wt/vol), vortexed and centrifuged at 1700 × g for 20 min. The supernatants were extracted with chloroform, vortexed, set at room temperature for 10 min and then centrifuged at 1700 × g for 20 min. The resulting aqueous supernatants were adsorbed with 10% dextran-coated charcoal, vortexed, centrifuged at 1700 × g for 20 min and the supernatant added to vials with scintillation cocktail and counted in a beta counter. The amount of radioactivity in water [^{3}H] measured was corrected by subtracting the blank values from each sample, obtained by incubating dishes containing medium with the tritiated androgen, but no cells. The values were also corrected by taking into account the fractional retention of tritium in medium water throughout the procedure of incubation and processing, utilizing parallel dishes containing medium plus known amounts of [^{3}H] water (NEN Life Science Products, Boston, MA, USA) through incubation and assay. The fractional retention of tritium in medium water throughout the incubation and processing of samples was always higher than 82%.

Measurement of aromatase mRNA

Analysis of the aromatase mRNA was carried out by reverse transcription PCR (RT-PCR) in MCF-7 cells. The total cellular RNA was purified with the Aurum™ Total RNA Mini Kit (Bio Rad Laboratories Inc., Hercules, CA, USA) following the manufacturer's instructions. Integrity of RNA was assessed by electrophoresis in ethidium bromide-stained 1.2% agarose-Tris-borate EDTA gels. The absorbance ratio A_{260nm}/A_{280nm} was greater than 1.8. For cDNA synthesis, 0.5 μg of total RNA were denaturated at 70 °C for 10 min and reverse transcribed 50 min at 37 °C with M-MLV Reverse

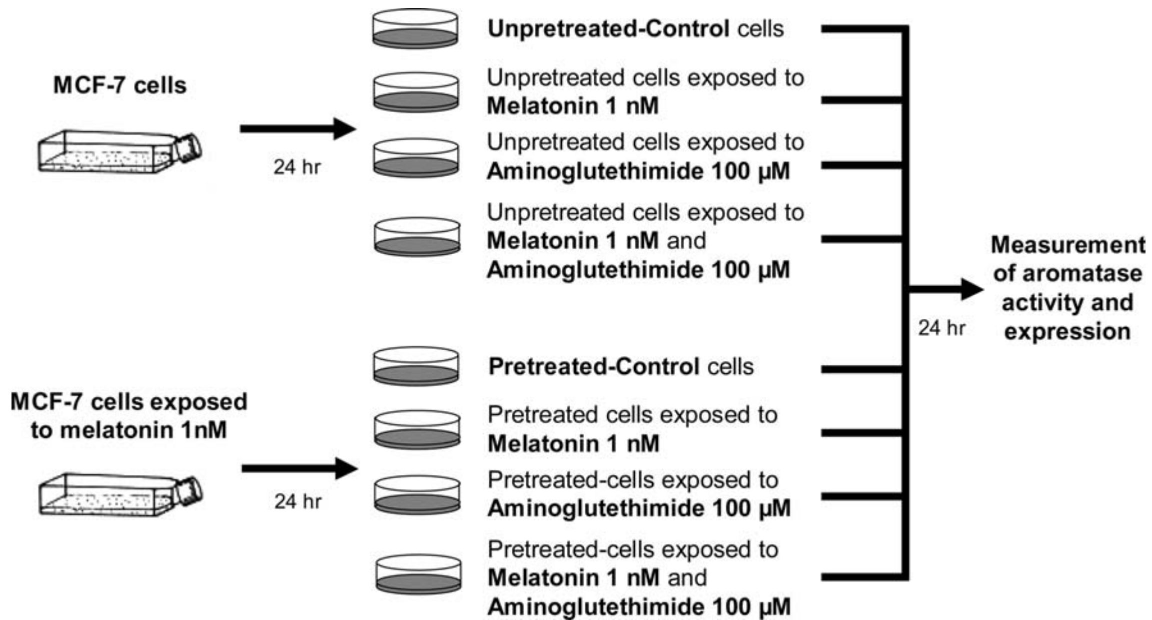

Figure 1. Flow diagram of the experimental design (see Material and methods).

Transcriptase (Invitrogen, Carlsbad, CA, USA) in a final volume of 20 μl in the presence of 500 ng of oligo (dT)12–18 primer.

PCR was performed using a set of human aromatase specific primers [5′–CAAGGTTATTTGATGCATGG (forward primer) and 5′-TTCTAAGGCTTTGCG CATGAC (reverse primer)] (Sigma Genosys Ltd., Cambridge, UK). The coding sequence between the two PCR primer sites is interrupted by three introns in the gene. As a control quantification, GADPH mRNA was also carried out by RT-PCR using a set of specific primers [5′-CCACCCATGGCAAATTCCATGGCA (forward primer) and 5′-TCTAGACGGCAGGTCA GGTCCACC (reverse primer)].

PCRs were performed for 34 cycles for semiquantitative analysis using the following temperature profile: 55 °C, 45 sec (annealing); 72 °C, 90 sec (extension); and 95 °C, 45 sec (denaturation). Each product was electrophoresed on ethidium bromide-stained 1.5% agarose-Tris-borate gels.

Statistical analysis

The data on aromatase activity are expressed as the mean ± standard errors of the mean (SEM). Statistical differences between groups were processed by one way analysis of variance (ANOVA) followed by the Student-Newman–Keuls test. Results were considered as statistically significant at $p < 0.05$.

Results

The aromatase activity of MCF-7 incubated for 24 h with tritiated androstenedione was estimated by the formation of tritiated water. As shown in Figure 2, aromatase activity of unpretreated MCF-7 cells was decreased by 24 h incubation with either 100 μM aminoglutethimide (55% of controls) or 1 nM melatonin (10% of controls). The effects of aminoglutethimide and melatonin were not additive, as coincubation of the unpretreated MCF-7 cells with both agents reduced the aromatase activity to a similar level as when cells were incubated with aminoglutethimide alone. However, a sequential regimen of melatonin (1 nM) followed 24 h later by aminoglutethimide (100 μM) resulted in an higher antiaromatase effect of this drug, significantly reducing ($p < 0.05$) aromatase activity of MCF-7 cells to values below those obtained in unpretreated cells and exposed to the same doses aminoglutethimide (Figure 2). Pretreatment of MCF-7 cells with 1 nM melatonin for 24 h before the exposition to the same concentration of melatonin, also induces a significant ($p < 0.05$) decrease in the cellular aromatase activity, higher than that previously obtained in unpretreated cells.

In a second experiment, cells, unpretreated or pretreated with 1 nM melatonin for 24 h, were incubated with 100 μM aminoglutethimide and/or 1 nM melatonin for 90 min and total RNA was isolated to perform semiquantitative RT-PCR with specific primers for human aromatase. Samples were taken between 28 and 32 cycles, in such a way that a linear relationship between PCR products and amplification cycles was observed. In unpretreated cells, aminoglutethimide did not modify aromatase RNA expression compared to control cells. Melatonin treatment inhibited aromatase mRNA expression in unpretreated cells. A sequential treatment of cells with melatonin followed by aminoglutethimide induced a significant inhibition in the aromatase mRNA expression. The chromatogram shown in Figure 3 pointed out the magnitude of the

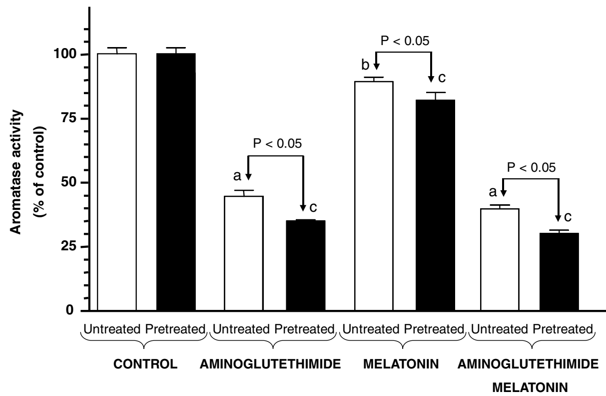

Figure 2. Effects of melatonin pretreatment on aromatase activity inhibitor actions of aminoglutethimide. MCF-7 cells were cultured in 75 cm^2 plastic culture flasks in DMEM supplemented with 10% FBS in the presence or not of melatonin (1 nM) for 24 hours. After this, cells cultured or not with melatonin were harvested and seeded onto 60×15 mm tissue culture dishes (1.5×10^6 cells/dish) in DMEM supplemented with 5% sFBS and incubated for 48 h. Then, medium was changed and replenished with fresh medium containing 100 nM [1β-^{3}H(N)]-androst-4-ene-3,17-dione] in the presence of either melatonin (1 nM) (M), aminoglutethimide (100 μM) (A), aminoglutethimide (100 μM) plus melatonin (1 nM) (AM) or the diluent (C) of these drugs. Aromatase activity was determined after 24 h of incubation, as described in material and methods. Data are expressed as the percentage of the untreated-control group (mean ± S.E.M.). a, $p < 0.001$ versus unpretreated-control cells; b, $p < 0.05$ versus unpretreated-control cells; c, $p < 0.001$ versus pretreated-control cells.

downregulation of aromatase expression induced by melatonin pretreatment. Both agents added simultaneously to pretreated cells showed an aromatase mRNA expression similar to pretreated cells exposed to only melatonin.

Discussion

The importance of estrogens in the development of breast cancer is well established from numerous experimental and clinical studies [16–18]. Ovaries constitute the main site of estrogen synthesis in the premenopausal non-pregnant woman. However, there are other local sources in some tissues, including skin, muscle, fat, benign and malignant mammary tissue, which acquire a special importance after menopausie [19]. In these peripheral tissues, estrogens (estrone and estradiol) are produced mainly through aromatization of adrenal androgen precursors (androstenedione and testosterone) and reach tissue concentrations higher than in plasma [20]. The principal enzyme responsible for the conversion of androgens to estrogens is a cytochrome P–450 complex known as aromatase [12,20]. At this moment, one of the main strategies for the design of anticancer drugs with estrogens as their target is based on the inhibition of estrogen synthesis by inhibiting the aromatase enzyme [21].

Melatonin exerts oncostatic effects on breast cancer, as described from *in vivo* and *in vitro* studies [4–7]. Much of the current knowledge about the mechanisms by which melatonin inhibits tumor cell growth points to an interaction of melatonin with estrogen-responsive pathways, thus behaving as an antiestrogenic hormone [7,22–24]. Our group has recently described that melatonin, within its antiestrogenic actions on breast cancer cells, reduces the aromatase activity and downregulates aromatase mRNA steady state levels in MCF-7 human breast cancer cells, that is, it behaves as a selective estrogen enzyme modulator by reducing the conversion of androgens to estrogens at tumor cell level [11]. Since both melatonin and aminoglutethimide have been shown to reduce tumor growth and tumor cell proliferation and decrease cellular aromatase activity [4,5,25], the aim of this study was to assess whether a sequential treatment with melatonin prior to aminoglutethimide increases the efficacy of the aromatase inhibitor used in treating breast cancer.

The present study demonstrates that a sequential treatment of MCF-7 cells with melatonin for 24 h followed by aminoglutethimide is the most effective way of reducing the aromatase activity in this tumor cell line. Aminoglutethimide action is more potent when MCF-7 cells are pre-exposed to melatonin rather than vehicle for 24 h. This finding suggests that melatonin sensitizes breast cancer cells so that aminoglutethimide becomes a more effective inhibitor of aromatase activity. Pretreatment of MCF-7 cells with melatonin also sensitizes tumor cells to the inhibitory effect of a subsequent re–exposure to melatonin, although this effect is more modest.

Subsequently, we evaluated the expression of aromatase mRNA steady state levels in MCF-7 and its possible modulation for both drugs. In unpretreated cells, aminoglutethimide did not modify aromatase RNA expression compared to control cells. While aminoglutethimide is effective in inhibiting aromatase activity, it has been described that aromatase expression cannot change or sometimes be increased after aminoglutethimide treatment both in patients and in cell culture experiments [13,26]. This finding may explain why some patients failed therapy after extensive aminoglutethimide treatment. It has also been suggested that a reduction of the circulating estrogen by aromatase inhibitor treatment leads to the release of gonadotropins from the pituitary of premenopausal women. These peptide hormones activate the mechanisms which increase the expression of aromatase in the peripheral tissue [13]. As expected, melatonin, in unpretreated cells also caused a reduction of aromatase mRNA expression; this result confirms those previously described by our group [11]. A sequential treatment of cells with melatonin for 24 h followed by aminoglutethimide induced a significant inhibition in the aromatase mRNA steady state levels as compared to cells exposed to the same doses of aminoglutethimide but unpretreated with melatonin. The chromatogram shown in Figure 3 pointed out the magnitude of the downregulation of aromatase expression induced by melatonin-pretreatment. Cells pretreated with melatonin and then re-

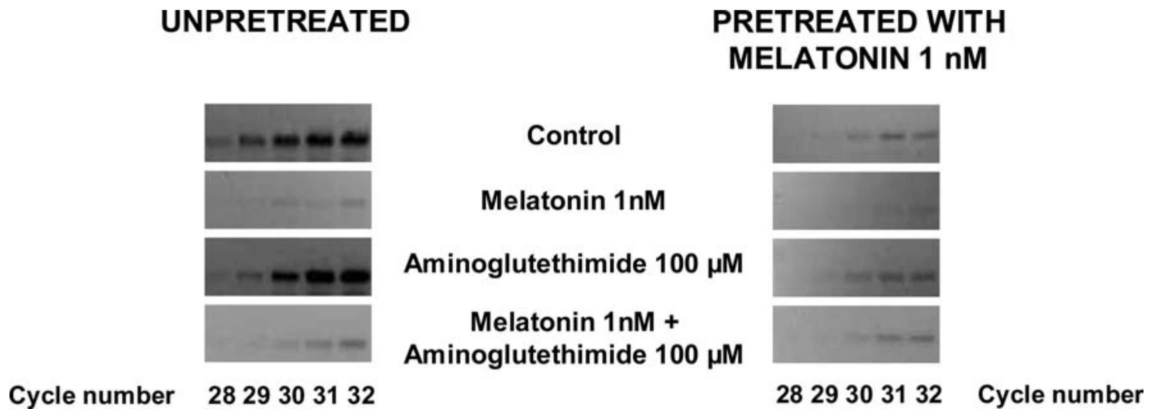

Figure 3. Effects of melatonin pretreatment on the expression of aromatase mRNA in MCF-7 cells. Unpretreated cells and cells pretreated with melatonin (1 nM), for 24 h, were then incubated with aminoglutethimide (100 μM) and/or melatonin (1 nM) for 90 min and total RNA was isolated to perform semiquantitative RT-PCR. cDNA was subjected to PCR using specific primers for P450 aromatase or GADPH. The results of a representative experiment (chromatogram analysis) are shown.

exposed to melatonin showed the lowest aromatase mRNA expression. These results suggest that the pretreatment with melatonin increases the reduction of the aromatase activity of cells exposed to aminoglutethimide as a result of the decrease in the aromatase mRNA expression induced by melatonin. Melatonin pretreatment reduces the aromatase expression in MCF-7 cells and when they are later exposed to a competitive aromatase inhibitor such as aminoglutethimide, that action reduces the amount of enzyme available and then increases the efficacy of this drug.

Aminoglutethimide modulation on aromatase expression is suppressed by adenylate cyclase inhibitors thus indicating that a cAMP-dependent mechanism might be involved [26]. In addition, in mammary cancer cells aromatase genes (CYP19) contain promoters II and I.3 regulated by cAMP [27–29]. Consequently, cAMP activates the transcription of aromatase promoters containing cAMP-responsive elements, while agents able to decrease cAMP could also decrease aromatase activity. Melatonin, through a membrane-bound Gi protein-coupled MT1 receptor, is reported to downregulate cAMP levels in a variety of cell types [30–32]. In MCF-7 cells, melatonin at concentrations of 10 nM or 1 μM, reduced the forskolin-induced increase of intracellular cAMP [32]. Our group [33] had previously demonstrated that melatonin induced changes in cyclic nucleotide synthesis in murine mammary glands. These changes consisted of a decrease in cAMP and an increase in cGMP accumulation, both responses being dose and time-dependent [33]. Thus, cAMP may be the link between melatonin, aminoglutethimide and aromatase activity and expression in human breast cancer cells.

The effects on aromatase mRNA expression of both melatonin and aminoglutethimide together both in unpretreated and in melatonin-pretreated cells were similar to those obtained with melatonin alone. This finding suggests that melatonin has a predominant and bigger effect on aromatase expression than aminoglutethimide. However, the effects of both drugs together on aromatase activity were similar to those obtained with aminoglutethimide alone, which suggests that this drug has a stronger effect on aromatase activity than melatonin. Although melatonin and aminoglutethimide have been shown to slow tumor proliferation and to decrease the aromatization of androgens at tumor level, they exhibit different modes of action. Aminoglutethimide is a reversible inhibitor which binds competitively to the heme moiety of aromatase, reduces aromatase activity and therefore decreases the local estrogen biosynthesis, but it has less important direct effects on aromatase RNA expression. However, melatonin reduces the aromatase activity primarily because it decreases aromatase mRNA steady state levels in MCF-7. The antiaromatase effects of melatonin pretreatment before aminoglutethimide are an example of additive actions of both hormones on the enzyme that change androgens into estrogens. The findings presented here point to melatonin pretreatment as a novel and interesting means to increase the efficacy of competitive aromatase inhibitors used in treating breast cancer. The development of treatments which would combine different pharmacological strategies, which would reduce the concentrations needed for clinical efficacy and enhance the oncostatic activity, would be of great benefit.

Acknowledgments

Supported by the Spanish MCYT (BFI2003-06305) and Institute of Health "Carlos III" (PI042603).

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