Antiestrogens modulate MT1 melatonin receptor expression in breast and ovarian cancer cell lines

OLIVER TREECK[1*], CHANDANA HALDAR[2*] and OLAF ORTMANN[1]

[1]Department of Obstetrics and Gynecology, University of Regensburg, Caritas Hospital St. Josef, Regensburg, Germany; [2]Pineal Research Laboratory, Department of Zoology, Banaras Hindu University, Varanasi-5, India

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Abstract. An interaction between cellular estrogen response and melatonin signaling mediated by G-protein coupled receptors is present in breast cancer cells. In this study, the effect of antiestrogens on basal and melatonin-modulated expression of MT1 melatonin receptor in breast and ovarian cancer cells was examined. For this purpose, the effects of the selective estrogen receptor modulator tamoxifen and pure antiestrogen ICI 182,780 on MT1 expression in estrogen receptor (ER) α-positive and -negative breast and ovarian cancer cell lines cultured in medium supplemented with 1 nM 17-ß estradiol were assessed by Western blot analysis. We were able to detect expression of the MT1 receptor in SK-OV-3 and OVCAR-3 cells and report its up-regulation by melatonin in both ovarian cancer cell lines. MT1 expression was observed to be significantly weaker in ERα-positive MCF-7 and OVCAR-3 cells than in ERα-negative MDA-MB-231 and SK-OV-3 cells. Treatment with the pure antiestrogen ICI 182,780 increased MT1 receptor expression in OVCAR-3 ovarian cancer cells, but decreased MT1 expression in MCF-7 breast cancer cells. No effect of ICI 182,780 on MT1 expression was observed in the ERα-negative cell lines SK-OV-3 and MDA-MB-231. After treatment with 4-OH tamoxifen, down-regulation of basal MT1 receptor expression in ERα-positive MCF-7 cells and inhibition of melatonin-induced up-regulation of MT1 in OVCAR-3 ovarian cancer cells were observed. In contrast, treatment with 4-OH tamoxifen increased the MT1 receptor level in ERα-negative SK-OV-3 ovarian cancer cells. Our findings support the existence of close interaction between estrogen and melatonin signaling. Moreover, our data suggest that melatonin signaling is modulated by antiestrogens in breast and ovarian cancer cells.

Correspondence to: Dr Oliver Treeck, Department of Obstetrics and Gynecology, University Regensburg, Caritas Krankenhaus St. Josef, Landshuter Str. 65, 93053 Regensburg, Germany
E-mail: otreeck@caritasstjosef.de

*Contributed equally

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Introduction

The pineal hormone melatonin affects cancer cells in various ways (1). *In vitro* experiments carried out with estrogen receptor (ER)-positive MCF-7 human breast cancer cells demonstrated that melatonin modulates the length of the cell cycle, increases expression of p53 and $p21^{WAF1}$ proteins (2), reduces metastatic capacity of these cells and counteracts the stimulatory effect of estradiol on cell invasiveness. This effect is mediated, at least in part, by a melatonin-induced increase in the expression of cell surface adhesion proteins E-cadherin and ß1-integrin (3). Furthermore, treatment with melatonin reduces the incidence of spontaneous mammary tumors in different strains of transgenic mice (c-neu and N-ras) and strains with a high tumor incidence (4,5).

The direct oncostatic effect of melatonin depends on its interaction with the cellular estrogen response. Melatonin impairs estrogen receptor signaling in breast cancer cells both by down-regulation of ERα expression and inhibition of ER-binding to DNA (6,7). Melatonin was demonstrated to be a specific inhibitor of estradiol-induced, ERα-mediated transcription and its mitogenic effects in both estrogen response element- and AP1-regulated promoters, whereas ERß-mediated transactivation was not inhibited or even activated at certain promoters. It has been demonstrated that the sensitivity of MCF-7 cells to melatonin depends on a high ERα/ERß ratio, and overexpression of ERß results in MCF-7 cells becoming insensitive to this hormone (8).

Different experimental studies suggest that estradiol stimuli modulate the cellular response to melatonin and regulate expression of melatonin receptors. Melatonin actions are mediated both by high affinity membrane receptors and nuclear receptors (9). MT1 membrane melatonin receptor belongs to the super-family of guanine nucleotide-binding regulatory protein (G protein)-coupled receptors. It was previously demonstrated that MT1 receptor expression in the rat ovary is down-regulated after estrogen exposure (10). In this study, we examined the effect of substances acting as inhibitors of estrogen receptor signaling on MT1 receptor expression in breast and ovarian cancer cells. Given that selective estrogen receptor modulators (SERMs) such as tamoxifen and pure antiestrogens like ICI 182,780 are important treatment options in hormone-dependent gynecological cancer, and antiestrogens are appropriate tools to further analyze the interaction between estrogen and

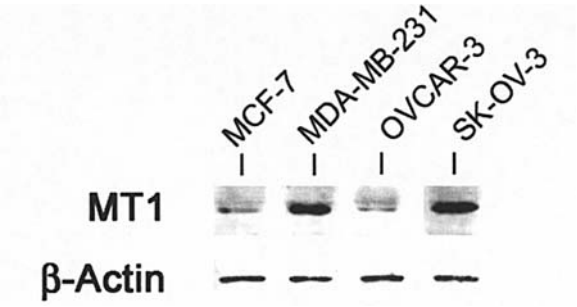

Figure 1. Western blot analysis of MT1 receptor expression in breast (MCF-7, MDA-MB-231) and ovarian (OVCAR-3, SK-OV-3) cancer cell lines. Protein (10 μg) of cell lysates prepared from cells cultured in 10% FCS was loaded and resolved by 10% SDS-PAGE. MT1 receptor was detected using the MT1 antibody N-20 (Santa Cruz Biotechnology), diluted 1:500. Detection of ß-actin expression was used as a loading control. Shown is a representative result from three independent experiments.

melatonin receptor signal transduction, we examined antiestrogen effects on melatonin signaling. For this purpose, the effects of tamoxifen and ICI 182,780 on basal and melatonin-modulated MT1 receptor expression were examined both in ERα-positive and -negative breast and ovarian cancer cell lines.

Materials and methods

Materials. Melatonin, 17-ß-estradiol and 4-OH tamoxifen were obtained from Sigma (Deisenhofen, Germany), and pure antiestrogen ICI 182,780 was purchased from Tocris (Bristol, UK). Fetal calf serum (FCS) was purchased from Invitrogen (Karlsruhe, Germany), and phenol red-free DMEM medium (with 1000 mg/l glucose and with L-glutamine) and serum replacement 2 (SR2) were obtained from Sigma (Deisenhofen, Germany). Cell Titer Aqueous One Solution Cell Proliferation (MTS) Assay was purchased from Promega (Madison, USA). MT1 antibody N-20 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), ERα antibody 62A3 from Cell Signaling Technology (Beverly, MA, USA) and ß-actin antibody 8226 was obtained from ABCAM (Cambridge, UK).

Cell culture and proliferation assays. MCF-7 and MDA-MB-231 breast cancer cell lines and OVCAR-3 and SK-OV-3 ovarian cancer cell lines (American Type Culture Collection, Manassas, VA, USA) were maintained in phenol red-free DMEM medium supplemented with 10% FCS, 1 nM 17-ß estradiol, 100 U/ml penicillin and 100 μg/ml streptomycin. For the serum-free culture, cell lines were cultured in DMEM containing the defined, estradiol- and growth factor-free serum replacement 2 (SR2; Sigma). All cell lines were cultured with 5% CO_2 at 37˚C in a humidified incubator. Before treatment with melatonin (± 1 nM 17-ß estradiol, depending on the experimental setup), cells were suspended in phenol red-free DMEM containing 10% FCS or 5% (v/v) steroid depleted charcoal-treated medium (SFCS) and seeded in 96-well plates (3-5x10^3 cells/well). Cells seeded in DMEM containing 10% FCS were stimulated with different concentrations of melatonin (1 and 100 nM) 24 h later. In the case of the serum-free culture, medium was changed from

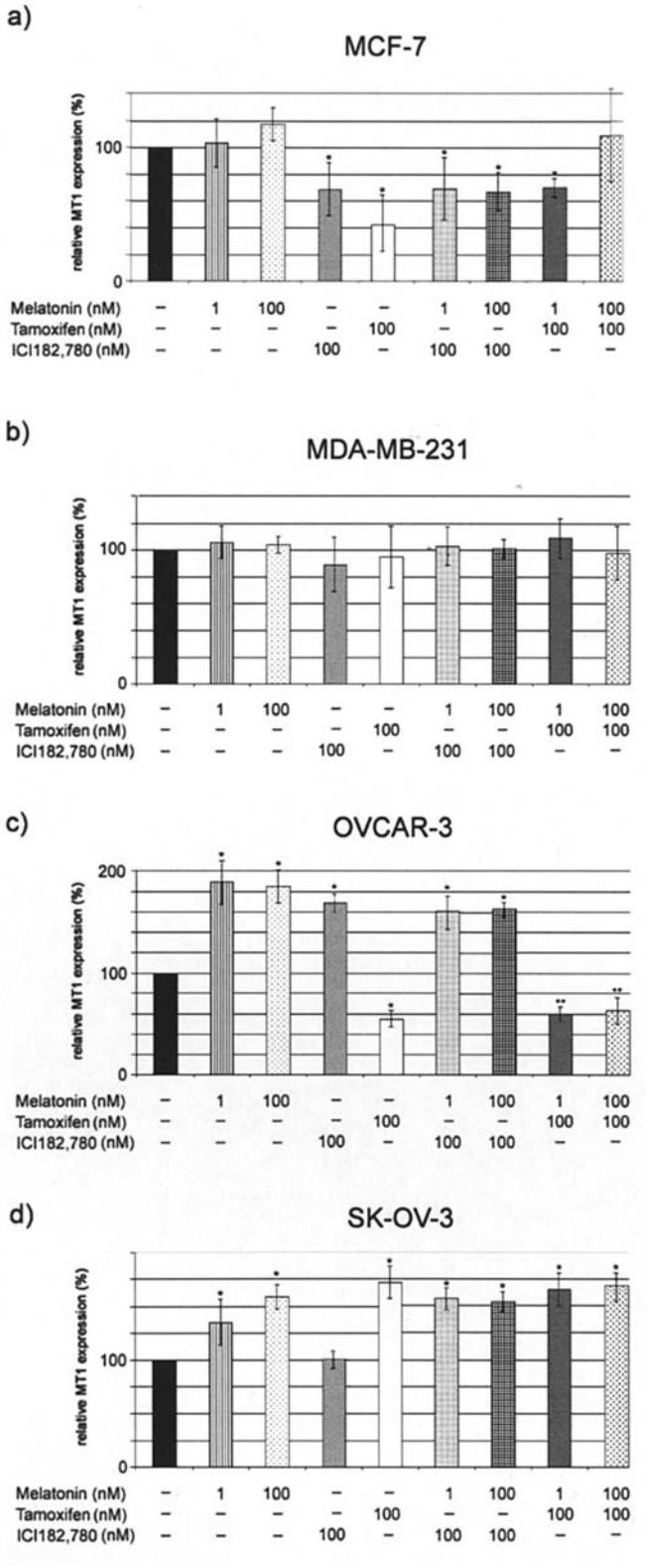

Figure 2. MT-1 receptor expression in breast and ovarian cancer cell lines after treatment with melatonin, 4-OH tamoxifen or ICI 182,780 analyzed by Western blot analysis. Cells cultured in DMEM supplemented with 10% FCS and 1 nM 17-ß-estradiol were treated with melatonin (1 and 100 nM) alone or in combination with 4-OH tamoxifen (100 nM) or ICI 182,780 (100 nM). After 48 h treatment, cells were lysed, and aliquots containing 10 μg protein were resolved by 10% SDS PAGE. Immunodetection was carried out using MT1 antibody (N-20; Santa Cruz Biotechnology), diluted 1:500 and ß-actin antibody (8226, ABCAM, Germany) diluted 1:2000. The quantification of Western blot band intensity, normalized to the ß-actin signal, are shown as the percentage of intensity in untreated cells (defined as 100%). Results were obtained from three separate experiments and expressed as means ± SD. *p<0.05 vs untreated control and **p<0.01 vs melatonin alone.

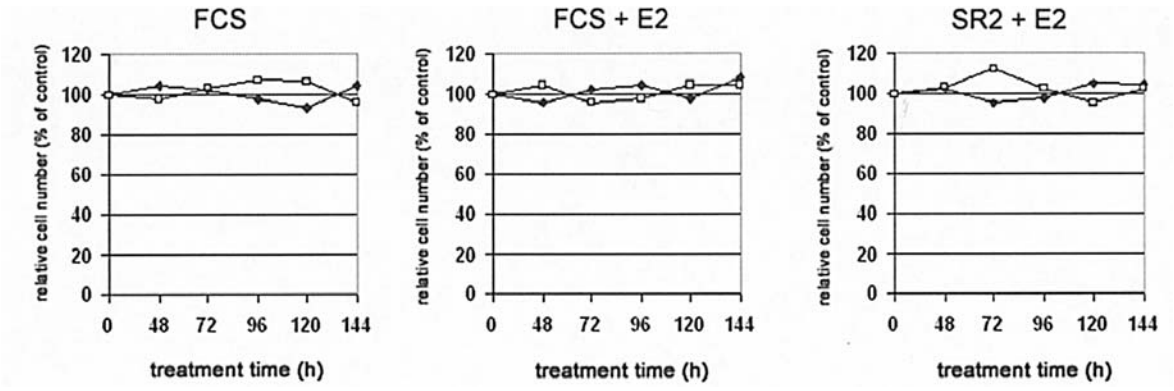

Figure 3. Effects of melatonin on growth of OVCAR-3 ovarian cancer cells. Cells grown in the indicated culture medium [FCS, DMEM supplemented with 10% FCS; FCS + E2, DMEM supplemented with 10% FCS; and 1 nM 17-ß-estradiol, SR2 + E2, DMEM supplemented with 1X serum replacement 2 (SR2) and 1 nM 17-ß-estradiol] were treated with different concentrations of melatonin (white square, 1 nM; black rhombus, 100 nM). After 48 and 96 h, treatment with melatonin was repeated. After the indicated time period, cells were subjected to the cell titer proliferation assay as described in Materials and methods. Results were obtained from 3 separate experiments and are expressed as the percentage of untreated control values (defined as 100%). Melatonin treatment of MCF-7, MDA-MB-231 and SK-OV-3 cells also did not affect cell growth under these experimental conditions (not shown).

5.0% to 0.5% (v/v) SFCS after 24 h, and cells were washed and incubated in DMEM-SR2 after 48 h, followed by treatment with test substances, in quadruplicate. After 48, 72 or 144 h treatment, cellular proliferation was quantified by measurement of relative cell numbers using the Cell Titer Aqueous Cell Proliferation (MTS) assay (Promega) according to the manufacturer's instructions or by use of a Neubauer improved counting chamber. Cell growth is expressed as the percentage of unstimulated medium control. The data obtained in 3 or 4 different experiments were pooled. After a Bartlett's test had shown that variances were homogeneous, analysis of variance (ANOVA) was carried out. Data were then analyzed for statistical significance of differences between individual groups using the Newman-Keuls test with Prism 2.0 software (GraphPad, San Diego, CA, USA) and statistical significance set at $p<0.05$.

Antibodies and Western blot analysis. Cells cultured in DMEM/10% FCS supplemented with 1 nM 17-ß estradiol were treated with melatonin (1 and 100 nM) alone or in combination with 4-OH tamoxifen (100 nM) or ICI 182,780 (100 nM). After 48 h treatment, cells were lysed in RIPA buffer [1% (v/v) Igepal CA-630. 0.5%(w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS) in phosphate-buffered solution (PBS) containing aprotonin and sodium orthovanadate]. Aliquots containing 10 μg protein were resolved with 10% (w/v) SDS-polyacrylamide gel electrophoresis, followed by electrotransfer to a PVDF hybond (Amersham, Buckinghamshire, UK) membrane. Immunodetection was carried out using MT1 antibody (N-20, diluted 1:500; Santa Cruz Biotechnology), ERα antibody (62A3; Cell Signaling, USA) and ß-actin antibody (8226; ABCAM, Germany) both diluted 1:2000 in PBS containing 5% skim milk (w/v) followed by horseradish peroxidase-conjugated secondary antibody (donkey and mouse), which were detected using chemiluminescence (ECL; Amersham). Bands were quantified by measurement of optical density using Scion Image analysis software (Scion Corporation, MD, USA). Values are expressed as the ratio of density of the specific signal to ß-actin signal. Data obtained in 4 different experiments were pooled. After a Bartlett's test had shown that variances were homogeneous, analysis of variance (ANOVA) was performed. The data were then analyzed for statistical significance of differences using the Newman Keuls test with Prism 2.0 software (GraphPad) and statistical significance set at $p<0.05$. The FCS + E2 and SR3 values were taken as 100% to calculate the percentage of expression of MT1 and ERα.

Results

To characterize our cell culture models of breast and ovarian cancer regarding their specific receptor status enabling a specific cellular response to melatonin, we performed Western blot analysis. We detected MT1 receptor protein in all cell lines cultured in media supplemented with 10% FCS and 1 nM 17-ß estradiol as a band of about 39 kDa. The strongest expression of MT1 was observed in ERα-negative MDA-MB-231 breast cancer and SK-OV-3 ovarian cancer cells. In ERα-positive MCF-7 breast cancer and OVCAR-3 ovarian cancer cells, a weaker expression of MT1 was detected (Fig. 1).

To examine the effect of antiestrogens both on basal and melatonin-modulated MT1 expression, we treated ERα-positive and -negative breast and ovarian cancer cell lines cultured in DMEM supplemented with 10% FCS and 1 nM 17-ß estradiol with melatonin alone or in combination with the selective estrogen receptor modulator (SERM) 4-OH tamoxifen and pure antiestrogen ICI 182,780. Treatment with melatonin (1 or 100 nM) resulted in the up-regulation of MT1 expression in ERα-positive OVCAR-3 and ERα-negative SK-OV-3 ovarian cancer cells. Treatment with 4-OH tamoxifen (100 nM) reduced the basal MT1 expression in MCF-7 breast cancer cells and both the basal and melatonin-induced expression of MT1 in OVCAR-3 ovarian cancer cells. In contrast, treatment with 4-OH tamoxifen increased MT1 receptor expression in SK-OV-3 ovarian cancer cells. No effect of 4-OH tamoxifen on MT1 expression was observed in ERα-negative MDA-MB-231 breast cancer cells. The pure antiestrogen and ERα down-regulator ICI 182,780 was able to reduce the basal MT1 expression in MCF-7 cells. In contrast, treatment with ICI 182,780 up-regulated MT1

receptor expression in OVCAR-3 ovarian cancer cells. No effects of this antiestrogen on MT1 expression could be detected in the ERα-negative breast and ovarian cancer cell lines MDA-MB-231 and SK-OV-3 (Fig. 2).

To determine whether inhibitors of estrogen signaling such as 4-OH tamoxifen or ICI 182,780 modulate not only melatonin signaling, but also melatonin effects on tumor cell growth, which were reported previously, we examined the effect of melatonin (1 and 100 nM) alone or in combination with 4-OH tamoxifen or ICI 182,780 on growth of ERα-positive MCF-7 and OVCAR-3 and ERα-negative MDA-MB-231 and SK-OV-3 cancer cell lines. Using a variety of experimental conditions, we observed no effect of melatonin on the growth of these tumor cell lines either when measured using the Cell Titer Aqueous One Solution Cell Proliferation (MTS) assay (Promega) or when cells were counted using a Neubauer improved counting chamber. Because melatonin effects on MCF-7 cell growth were previously reported, we changed the experimental conditions regarding the cell culture medium [with 10% FCS or serum-free, both ± supplementation with 10 nM 17-ß-estradiol (E2)], melatonin concentration (1 and 100 nM) and stimulation time (48 to 144 h). None of these experimental set-ups resulted in the detection of an antiproliferative effect of melatonin on breast and ovarian cancer cells in our *in vitro* systems (Fig. 3).

Discussion

Human MT1 melatonin receptor is a high affinity G protein-coupled membrane receptor, its mRNA encodes 350 amino acids with a predicted molecular mass of 39 kDa (11). Expression of MT1 receptor has been previously demonstrated in MCF-7 breast cancer cells (12). In this study, we additionally report expression of MT1 in MDA-MB-231 breast cancer cells and OVCAR-3 and SK-OV-3 ovarian cancer cells. Interestingly, levels of MT1 expression were significantly higher in ERα-negative SK-OV-3 and MDA-MB-231 cells than in ERα-positive MCF-7 and OVCAR-3 cells, supporting previous studies reporting an inverse correlation between the expression of both receptors resulting from the down-regulation of melatonin receptors by estrogens or the down-regulation of ERs by melatonin. Previous studies also reported an inverse correlation between melatonin levels and ERα expression. Nanomolar concentrations of melatonin were demonstrated to decrease transcription of the ERα gene, resulting in suppression of both ER mRNA and protein levels in a time-dependent manner (13).

The effect of melatonin on tumor cell growth *in vitro*, however, remains controversial. In some studies, melatonin was observed to inhibit growth of human MCF-7 breast cancer cells at physiological concentrations of 1 nM *in vitro* (14). In our study, we found no inhibitory effect of melatonin (1 and 100 nM) on the growth of breast or ovarian cancer cells growing in culture medium supplemented with 1 nM 17-ß estradiol irrespective of their ERα receptor status. We did not observe any melatonin effect, even though we modulated the experimental conditions in various ways (e.g. using different melatonin incubation times from 48 to 144 h, repeating melatonin stimulation every 24 h, or using different cell culture medium containing 10% FCS or serum-free medium). Thus, our data support the results of previous studies reporting no effect of melatonin on MCF-7 or OVCAR-3 cancer cell growth *in vitro* (15-17). On the other hand, a growth inhibitory effect of melatonin on MCF-7 breast cancer cells has been reported by studies that specifically used synchronized cells (6). We decided to not synchronize growth of our cell culture models because tumor cells *in vivo* do not grow synchronized; therefore, we cannot rule out that the inhibitory effect of melatonin on the cell cycle previously reported was present, but undetectable under our experimental conditions.

The molecular mechanisms underlying the interaction of melatonin and estrogen signaling are not fully understood. Evidence indicates that melatonin does not bind to ER or interfere with the binding of estradiol to its receptors, but is able to impair DNA binding of ER (7,18). On the other hand, it has been demonstrated that the cellular response to melatonin is modulated by estrogen signaling. Data suggest that estradiol stimuli regulate the expression of melatonin receptors. It has been previously demonstrated that MT1 receptor expression in the rat ovary is down-regulated after estrogen exposure (10). Furthermore, the antiestrogen ICI 164,384 inhibits melatonin-triggered transactivation of ERα, mediating melatonin signaling in MCF-7 cells (19). Here, we report an additional molecular mechanism by which antiestrogens modulate melatonin action: the regulation of MT1 receptor expression.

Our data, generated from experiments with breast and ovarian cancer cell lines cultured in DMEM supplemented with 10% FCS and 1 nM 17-ß estradiol, demonstrated an increase of MT1 protein expression in ovarian cancer cells after treatment with 1 or 100 nM melatonin for 48 h. This effect was more pronounced in ERα-positive OVCAR-3 cells, and also present in SK-OV-3 cells not expressing functional ERα, suggesting that the melatonin-induced MT1 up-regulation is an effect that is at least partially independent of ERα signaling. The different antiestrogen effects on both ovarian cell lines suggest a different role of estrogen receptor activation in melatonin signaling of SK-OV-3 and OVCAR-3 cells. Treatment with the pure antiestrogen ICI 182,780 increased MT1 expression in ERα-positive OVCAR-3 cells, whereas no such increase was observed in ERα-negative SK-OV-3 ovarian cancer cells, suggesting that this effect is ERα-dependent. Whereas 4-OH tamoxifen decreased basal and melatonin-induced MT1 expression in ERα-positive OVCAR-3 ovarian cancer cells, this drug increased the MT1 protein level in ERα-negative SK-OV-3 ovarian cancer cells. Since a decrease of MT1 protein level after treatment with 4-OH tamoxifen is only observed in MCF-7 and OVCAR-3 cells, this effect may be dependent on the expression of functional ERα, which is not present in MDA-MB-231 and SK-OV-3 cells. The differences between the effects of 4-OH tamoxifen and ICI 182,780 on MT1 expression in our tumor cell lines could be explained by their different mechanism of action. Binding of the pure antiestrogen ICI 182,780 to ERs leads to a protein conformation that does not allow the dimerization of two ERs, resulting in increased protein degradation. In contrast, tamoxifen binding to ERs does not disable dimerization and cofactor binding of ER, but modulates the transcriptional activity of ERs. Furthermore, this drug exerts

nonspecific effects on other cellular signaling pathways. Thus, ICI 182,780 is a more specific tool to examine the role of estrogen signaling in melatonin response. Since previous reports demonstrated down-regulation of the MT1 receptor after stimulation with estradiol in different experimental models, the increase of MT1 protein level after ICI 182,780 treatment in OVCAR-3 cells may be a specific effect antagonizing the suppression of MT1 receptor by estradiol present in our experimental system. Given that increased expression of MT1 melatonin receptor has been demonstrated to both enhance melatonin signaling and intensify the antiproliferative action of this hormone (20), it is tempting to speculate that antiestrogens may modulate antitumoral melatonin action in a cell type-specific manner. Although we did not measure the effect of melatonin on unsynchronized growth of the employed cancer cell lines using different culture conditions, and thus did not succeed in examining the physiological effects of the observed MT1 receptor regulation, our findings indicate that melatonin receptor expression is modulated by antiestrogens in breast and ovarian cancer cells.

In conclusion, this study is the first to report on the expression of MT1 receptor in SK-OV-3 and OVCAR-3 cells and its up-regulation by melatonin in both ovarian cancer cell lines. Furthermore, our data support previous studies reporting a close interaction between melatonin and estrogen receptor signaling. Further studies are needed to elucidate the consequences of antiestrogen-triggered regulation of MT1 receptor expression.

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