SENSITIZING EFFECTS OF MELATONIN ON DOCETAXEL TREATMENT IN HUMAN BREAST CANCER CELLS (MCF-7)

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INTRODUCTION

- **Breast cancer** is the most common cancer worldwide. The pathogenesis and growth of breast cancer are linked to estrogens, which are considered key molecules in mammary carcinogenesis. About 2 of 3 breast cancers are estrogen receptor-positive (ER+) and, therefore, they respond to hormonal therapy.
- **Docetaxel**, a microtubule inhibitor agent (MIA), is a spindle poison commonly used in breast cancer chemotherapy treatment. The main mechanism of its cytotoxicity is due to disruption of microtubule function, particularly of microtubules comprising the mitotic spindle apparatus, directly causing metaphase arrest.
- **Melatonin**, the pineal hormone, has been shown both *in vivo* and *in vitro* to have an oncostatic role on ER+ tumors. This hormone has anti-estrogenic properties, inhibiting estrogen synthesis behaving as a selective estrogen enzyme modulator (SEEM) and also interferes with estrogen signaling, behaving as a selective estrogen receptor modulator (SERM). Clinical trials conclude that patients who received melatonin showed substantial improvements in tumor remission, survival and side effects of chemotherapy, which points to melatonin as a potential adjuvant for chemotherapy to be considered.
- **Chemotherapy** presents some problems such as side effects, development of resistance or changes in gene expression of cells. In the past few years, evidence of a broader spectrum of action of melatonin as an antitumoral agent have arisen, thus, melatonin seems to have positive effects in several types of cancer, counteracting the undesired effects of chemotherapy.

HYPOTHESIS AND OBJECTIVES

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

- **To establish the changes in gene expression induced by docetaxel.**
- **To verify if melatonin is able to modulate those changes.**
- **To check if melatonin potentiates the antiproliferative effects of docetaxel.**
- **To test the ability of melatonin to further stimulate the apoptosis triggered by docetaxel.**

MATERIALS AND METHODS

- **Cell line, culture conditions and treatments**: MCF-7 cells derived from human mammary adenocarcinoma (ER+) were used in this work. Cells were maintained in Petri dishes at 37°C in a humid atmosphere containing 5% CO_2 in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal bovine serum (FBS). Antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) were added to protect the cells from contamination. Cells were treated with different concentrations of docetaxel (ranging from 1 µM to 0.01 nM) and/or melatonin (1 nM). Melatonin pretreatment consisted in addition of melatonin (1 nM) 24 hours prior to other treatments.
- **Study of gene expression**: After four hours of treatments, total RNA was isolated and purified with the NucleoSpin II (Macherey-Nagel) commercial kit. The absorbance ratio A260/A280 was greater than 1.9. For cDNA synthesis, 500 ng of total RNA was used as template using the RT² First Stand Kit (Qiagen). Pathway-focused gene expression profiling was performed using a 96-well human breast cancer PCR array (RT² Profiler PCR array, Qiagen), monitoring the expression of 84 genes involved in tumor classification, signal transduction, angiogenesis, adhesion, proteolysis, cell cycle and apoptosis. The array includes 5 housekeeping genes which expression is not influenced by the experimental conditions.
- **Expression of p53, p21, CDH13, BAX, BAD, BCL-2, MUC1, GATA3 and MYC**: Specific analysis of mRNA expression of these genes was carried out by RT-PCR in an MX3005P (Agilent) using RT2 SYBR Green qPCR MasterMix (Qiagen). β-actin was used as a housekeeping gene. The primers were designed so that the coding sequence between the two PCR primer sites is interrupted by at least one intron in the gene. Amplifications were as follows: 1 cycle at 95°C for 10 minutes and 45 cycles for quantitative analysis using the following temperature profile: 95°C for 30 seconds and 60°C for 60 seconds. Melting curves were performed to verify that only a single product with no primer-dimers was amplified. Each reaction was run at least in triplicate.
- **Measurement of cellular proliferation**: MCF-7 cells were seeded into 96-well plates at a density of 4000 cells per well in DMEM supplemented with 10% FBS. After 24 hours of incubation, media containing different concentrations of docetaxel and/or melatonin or vehicle (ethanol) were added. Cell proliferation was measured by the MTT method.
- **Determination of apoptosis**: Cells were seeded into 6-well plates in DMEM supplemented with 10% FSB. After 24 hours, media containing 10 nM docetaxel, 1 nM melatonin or both was added. Induction of apoptosis was determined using an Annexin V-FITC apoptosis detection kit (Miltenyi Biotec, GmbH). The results were analyzed by flow cytometry (Becton Dickinson FACS CANTO II analyzer) using the FL-1 (green; annexin V-FITC) and FL-3 (red; PI) detectors. Under all conditions tested, the percentages of annexin$^+$/PI$^-$(early apoptotic) and annexin$^-$/PI$^-$ cells (viable cells) were mainly compared.
- **Statistic analysis**: Results are expressed as the mean ± standard error of the mean (SEM). Statistical differences between groups were processed by One Way Analysis of Variance (ANOVA) followed by the student-Newman-Keuls test. Results were considered as statistically significant at $p < 0.05$. Data were expressed as percentage of the control group (mean ± SEM). In the figures, letters a, b and c represent the significance level over control groups; (a) $p < 0.05$ (b) $p < 0.01$ (c) $p < 0.001$. Letters d and e represent the significance level versus docetaxel 1 nM groups; (d) $p < 0.01$ (e) $p < 0.001$ and f represents the significance level versus the 0.1 nM docetaxel groups (f) $p < 0.001$.

RESULTS AND DISCUSSION

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

Table 1: Gene expression profiling (RT² Profiler PCR array). Up and down-regulated genes (more than 50% relative to control) when MCF-7 cells were treated with docetaxel (1 µM) and/or melatonin (1 nM).

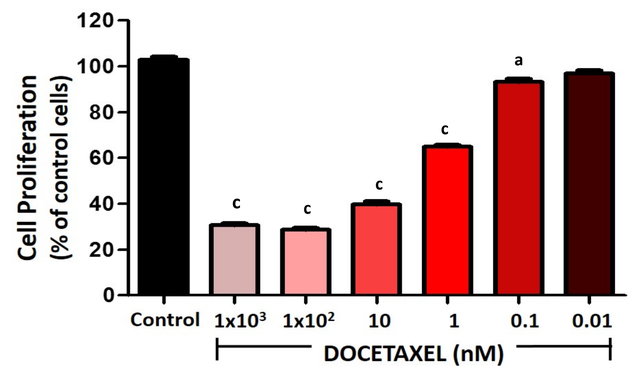


Figure 2: Effects of docetaxel dose on MCF-7 cell proliferation. Cells were treated with docetaxel at different concentrations for 3 days. Data are expressed as percentage of the control group (mean ± SEM).

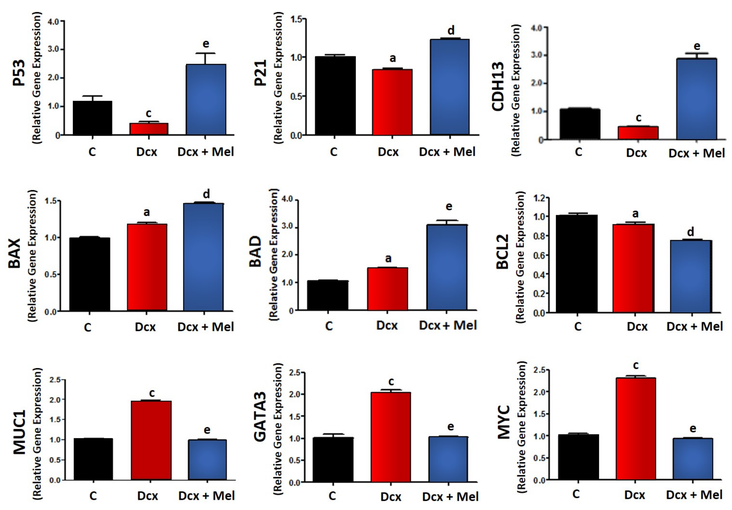


Figure 1: Expression of the genes p53, p21, CDH13, BAX, BAD, BCL-2, MUC1, GATA3 and MYC in MCF-7 cell line. Cells were treated with docetaxel (1 µM) and/or melatonin (1 nM).

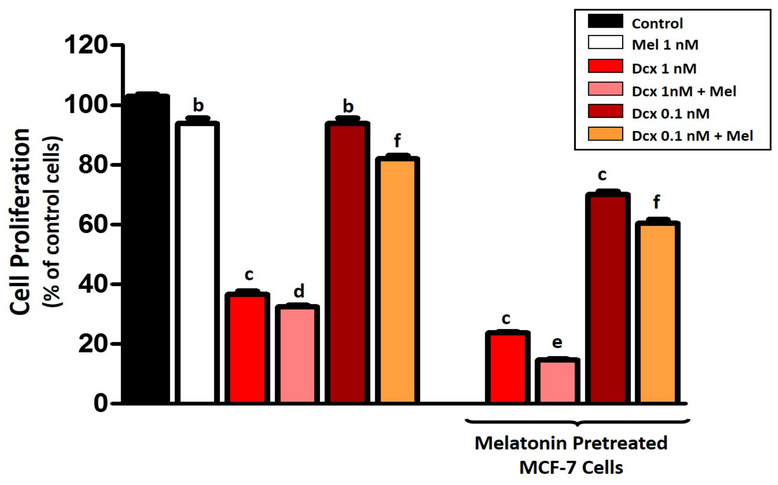
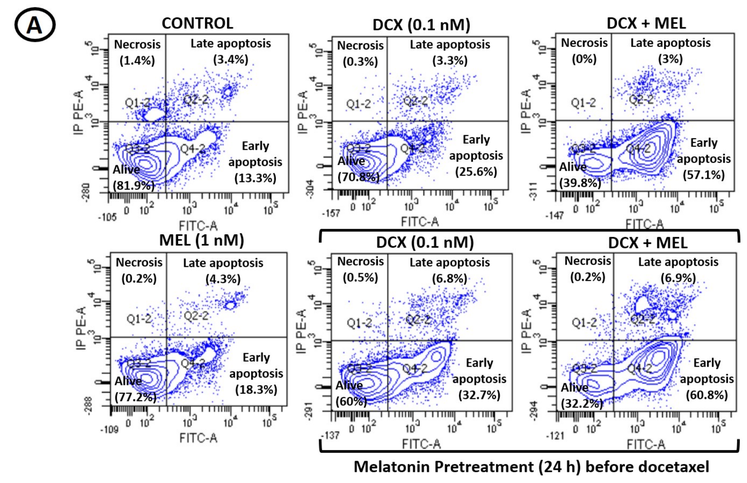


Figure 3: Potentiation of docetaxel-induced growth inhibition by melatonin. Effects of docetaxel (1 nM, 0.1 nM) and/or melatonin (1 nM) on MCF-7 cells proliferation after 6 days of culture. Data are expressed as percentage of the control group (mean ± SEM).



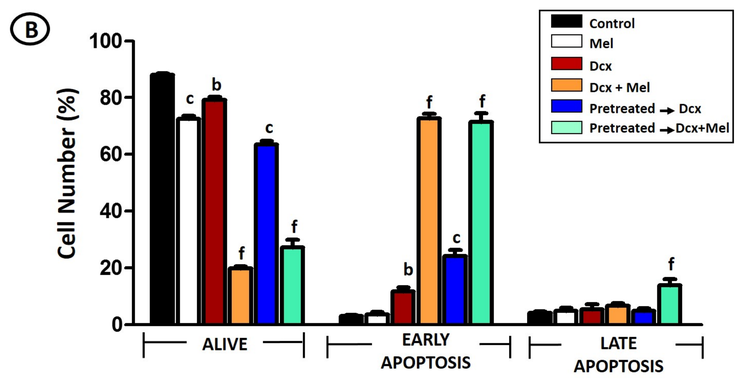


Figure 4: Potentiating effect of melatonin on docetaxel-induced apoptosis in MCF-7 cells. A) Representative dot-plots showing viable cells (Annexin$^-$/IP$^-$), early apoptotic (Annexin$^+$/IP$^-$) and late apoptotic (Annexin$^+$/IP$^+$) after 24 h of treatment with docetaxel (0.1 nM) and/or melatonin (1 nM) **B)** Histograms showing percentages of each population. Values are presented as means ± SEM.

1.- STUDY OF GENE EXPRESSION

1.1.- Docetaxel induced changes in the expression of many genes known to be deregulated in breast cancer and we demonstrate a modulatory effect of melatonin in the expression of 9 of these genes.

1.2.- Docetaxel reduced the expression of tumor suppressor genes p53 and p21 and cadherin 13 (silenced in many tumors). Melatonin counteracted this effect of docetaxel, even over control levels.

1.3.- Docetaxel slightly increased the expression of proapoptotic genes BAX and BAD, and reduced the expression of antiapoptotic BCL-2 gene. Melatonin maximized these effects.

1.4.- Docetaxel increased expression of the transcription factors MYC, MUC1 and GATA3 expression. This effect was partially counteracted by melatonin.

2.- MEASUREMENT OF CELLULAR PROLIFERATION

2.1.- Docetaxel inhibited MCF-7 cell proliferation in a dose dependent manner. Concentrations below 0.1 nM did not show antiproliferative effects. Concentrations equal or higher than 10 nM achieved maximum antiproliferative activity.

2.2.- Concomitant treatment with melatonin (1 nM) further stimulated the antiproliferative activity of subpharmacological doses of docetaxel (0.1 nM and 1 nM) in MCF-7 cells.

2.3.- Pretreatment with melatonin (applied 24 hours before adding docetaxel) sensitized MCF-7 cells to subpharmacological doses (0.1 nM and 1 nM) of docetaxel. Again, cotreatment with melatonin (1 nM) potentiated the antiproliferative activity of docetaxel.

3.- DETERMINATION OF APOPTOSIS

3.1.- MCF-7 cells treated with physiological concentrations of melatonin (1 nM) displayed a significant decrease in the percentage of alive cells, thus slightly increasing the proportion of early apoptotic cells.

3.2.- Treatment with docetaxel (0.1 nM) caused also a significant increment in the percentage of early apoptotic cells, at the expense of the amount of alive cells.

3.3.- Interestingly, simultaneous treatment with docetaxel and melatonin brought a further increase in the number of cells that enter into early apoptosis, thus improving the tumor killing efficacy of this chemotherapeutic agent.

3.4.- Pretreatment with melatonin for 24 hours prior docetaxel also enhanced the pro-apoptotic effect of this chemical agent.

CONCLUSIONS

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

FURTHER RESEARCH

- To test the modulatory effect of melatonin over the docetaxel altered expression of other genes of the list.
- To search for posttranslational modifications (cell-signaling pathways) triggered by docetaxel characterizing the possible role of melatonin as a modulator of those changes.
- To screen the changes in gene expression, proliferation and posttranslational modifications induced by other chemotherapy agents used in breast cancer treatment, such as vinorelbine, doxorubicin, trastuzumab, in presence or absence of melatonin.
- To screen the changes in gene expression, proliferation and posttranslational modifications induced by radiotherapy, checking the effect of melatonin modulating those changes.

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