Differential regulation by melatonin of cell growth and androgen receptor binding to the androgen response element in prostate cancer cells

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Abstract

OBJECTIVES: The pineal hormone melatonin inhibits the growth of benign human prostate epithelial cells and the androgen-dependent prostate cancer LNCaP cells. In the androgen-nonresponsive prostate carcinoma PC3 cells melatonin inhibits cell growth only at high but not low cell density. We have recently found that melatonin causes nuclear exclusion of the AR and attenuates it transcriptional activity in LNCaP cells as well as PC3 cells stably transfected with a wild type AR expressing vector (PC3-AR). The aim of this study was to investigate whether melatonin inhibits effects of AR on cell growth in PC3-AR cells and whether inhibition of AR DNA binding is involved.

METHODS: The effects of androgen, melatonin and their combination on the growth of the PC3-AR cells and on AR DNA binding in PC3-AR and LNCaP cells were studied.

RESULTS: DHT suppressed cell growth in the PC3-AR cells and enhanced AR binding to the androgen responsive element (ARE). Melatonin had no effect on cell growth in the absence of DHT but counteracted the androgen-induced inhibition at low androgen concentrations. Melatonin did not suppress and even slightly enhanced the capacity of AR binding to the ARE in the PC3-AR as well as in LNCaP cells.

CONCLUSIONS: Attenuation by melatonin of AR activity in the prostate cancer cells is not due to suppression of AR binding to the ARE, and is presumably caused by its effects on AR protein interaction and intracellular trafficking.

Introduction

Melatonin, the hormone secreted at night by the pineal gland, inhibits the growth of human benign (BPH) and tumor (LNCaP) prostate epithelial cells *in vitro* [1-4]. Melatonin inhibits growth of the prostatic carcinoma PC3 cells which do not express the androgen receptor (AR) only at high cell density and slightly enhances growth at low cell density [5]. The androgen receptor (AR) is an intracellular receptor that functions as a ligand dependent transcription factor [6]. The AR mediates all the activities ascribed to androgens, including development, differentiation and maintenance of male reproductive function, support of sexually dimorphic non-reproductive functions and pros-

tate cancer growth. In androgen target tissues, the AR is localized within the nucleus [7]. Upon ligand binding, the AR binds to hormone response elements in the promoter region of inducible genes thus controlling their transcription [6]. Mutations at the DNA binding domain of the AR, leading to nuclear exclusion of the receptor and loss of androgen sensitivity, have recently been identified in prostate cancer cells [8].

We have therefore sought to investigate whether the introduction of the AR into PC3 cells will confer melatonin-mediated effects on AR-dependent growth in PC3 cells. For this purpose we used PC3-AR cells, which are derived from the AR-negative prostatic carcinoma PC3 cells by stable transfection with an AR expressing vector [9]. In the PC3-AR cells as well as the androgen responsive prostate cancer LNCaP cells, melatonin causes nuclear exclusion of the AR and attenuates androgen induced gene expression [10, unpublished data]. Melatonin does not inhibit androgen binding in these two cell types and up-regulates AR level [10, unpublished data].

One possible explanation for a melatonin-mediated attenuation of AR effects and nuclear exclusion would be a decrease in AR binding to its DNA response element (ARE). Hence, the effects of melatonin on AR binding to the ARE were studied. Because the AR in PC3 cells is not an innate receptor to these cells, the effects of melatonin on AR-DNA binding was also studied LNCaP cells that express an innate mutant AR that has a high affinity and low selectivity towards sex steroids [11].

Materials and methods

PC3-AR Cell culture: Human PC3 cells stably transfected with wild-type AR cDNA [9] were donated by Dr A Cato (Karlsruhe, Germany). Human LNCaP prostate cell line were obtained from American Type Culture Collection (MA, USA). The cells were cultured in RPMI-1640 medium (RPMI) medium containing 10% fetal calf serum (FCS), 50 units/ml penicillin, 50 µg/ml streptomycin (all from Beit Haemek, Israel). For each experiment the cells were plated (100,000 cells/well) in 6 well-dishes, in RPMI without phenol red containing 2 mM glutamine, 5% charcoal-stripped FCS for 4 days. Medium was replaced every 2 days. Dihydrotestosterone (DHT; $10^{-12} – 10^{-7}$ M), melatonin ($10^{-10} – 10^{-7}$ M) their combination or vehicle were then added and incubation resumed for 1 or 24 h at 37°C in humidified atmosphere with 5% CO_2.

3H-Thymidine incorporation: Cells were incubated in culture medium in the absence or presence of melatonin for 60 min. at 37°C in 5% CO_2 atmosphere, 100% humidity with ^{3}H-thymidine (60 Ci/mmol, 1µCi/well). Media were discarded, and the cells fixed in methanol at –20°C for 15 min., rinsed with 2X2 ml PBS and then incubated 3X10 min with 2 ml ice-cold 10% trichloroacetic acid. Cell monolayers were then solubilized in 0.4 ml 0.3N NaOH solution containing 0.1% sodium dodecyl sulfate (SDS). The acid-precipitable material was counted for incorporated radioactivity in a β counter. Aliquots were retained for protein determination.

DNA determinations: Cells were washed with PBS and suspended in 0.5–0.7 ml of 0.2% SDS in ETN buffer (composed of 10 mM ethyleneglycoltetraacetate, 10 mM Tris Hcl, pH 7.0). The cell suspensions were incubated for 30 min. at 37°C to solubilize the cells. Aliquots (35–70 µl) of the solution were added to 1 ml of ETN buffer containing 1 µg/ml Hoechst 33258 (Sigma St Louis Mo) and RNase (5 ng/ml) and incubated in the dark for 30 min. at room temperature. The fluorescence of the dye at 450 nm wavelength (excitation at 360 nm) was then assessed. Calf thymus DNA was used as a standard.

Flow Cytometric Analyses: Cells (approximately $4X10^5$) were detached (by 0.5 mM EDTA) from plates and centrifuged (200 g). Each cell pellet was resuspended in 1 ml of hypotonic flurochrome solution (50 mcg/ml propidium iodide in 0.1% sodium citrate containing 0.1% Triton X-100) and stored overnight in the dark at 4°C prior to flow cytometric analysis as described [12]. Propidium iodide fluorescence was measured using FACSort flow cytometer (Becton Dickinson. Mountain View, CA). Excited at 488 nm and collected through a 570 nM BP filter. Data were analyzed using Mplus computer software.

Electrophoretic mobility shift assay: Whole cell extracts were prepared as described [13]. The binding reaction mixture containing 15000 cpm of ^{32}P-labeled double stranded AR DNA binding consensus sequence (Promega, Madison WI, U.S.A.)

5′-GTAGAAGTCTGGTAAGGGTTTCTTTTTGCA-3′
3′-GATCTTCAGACCATGTCCCACAAGAAAAACGT-5′
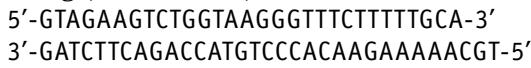
10 mM Tris-HCl (pH 7.9), 60 mM KCl, 0.4 mM dithiothreitol, 10% glycerol, 2 μg of bovine serum albumin, 1 μg of poly(dI-dC), was incubated for 30 min with 5 µg nuclear extract. For specificity control, a 50-fold excess of unlabeled probe was applied. Products were analyzed on a 5% acrylamide gel made up in 1xTGE (50 mM Tris, 400 mM glycine, 2 mM EDTA). Dried gels were exposed to X-ray film or to phosphor screen (Molecular Dynamics). Quantitative data were obtained using phosphoimager analysis (Molecular Dynamics).

Statistical Analyses: Results were compared by Analysis of Variance (ANOVA) followed by paired Student's t-tests. Significance was determined at p<0.05.

Results

Androgen suppresses PC3-AR cell growth, melatonin partially negates this effect

Thymidine incorporation: The effects of 1 and 24 h incubation with various concentrations of DHT, melatonin and their combinations on ^{3}H-thymidine incorporation into the PC3-AR cells are presented in Fig. 1. Androgen (1 and 24 h) markedly inhibited thymidine incorporation into the cells in a dose dependent manner with 50% inhibition (IC_{50}) around 1 nM.

Melatonin (10^{-7} but not 10^{-9} M) slightly inhibited ^{3}H-thymidine incorporation into the PC3-AR cells in the absence of DHT, whereas in the presence of the androgen, melatonin abrogated the suppression of ^{3}H-thymidine incorporation effected by low (10^{-12} M) DHT concentra-
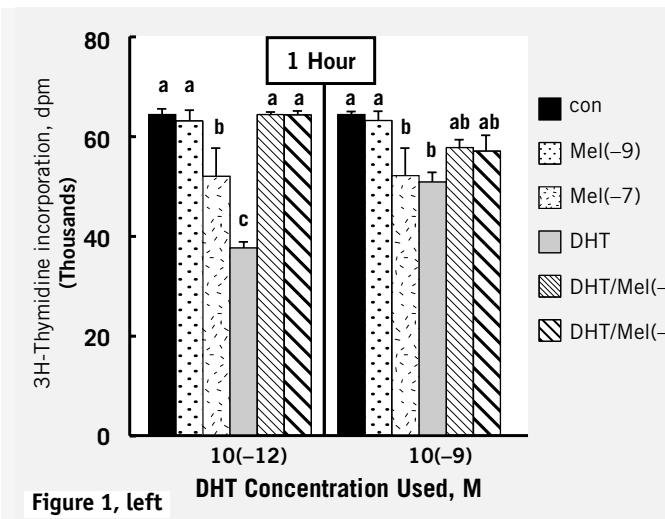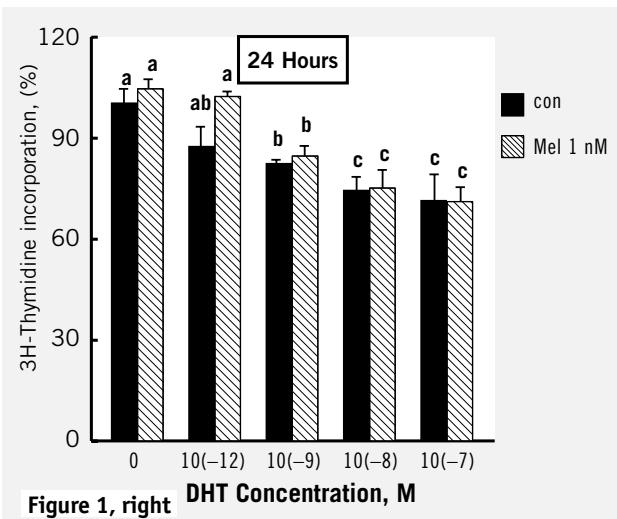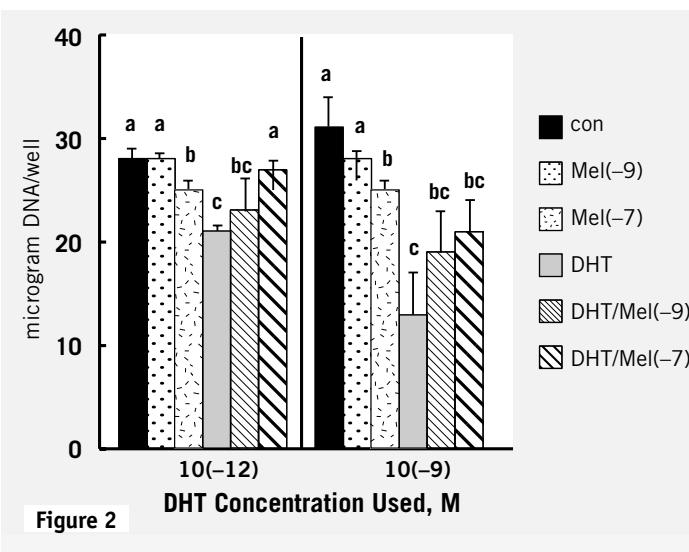
Figure 1, left

Figure 1, right

Figure 2

Fig 1: Effects of androgen, melatonin and their combinations on ^{3}H-thymidine incorporation in PC3-AR cells. **Left Panel:** Cells were treated with various concentrations of DHT (10^{-12}; 10^{-9} M) in the absence or presence of melatonin (10^{-9}; 10^{-7} M) for 1 h. **Right panel:** Cells were treated with various concentrations of DHT (0-10^{-8} M) in the absence (black bars) or presence (striated bars) of melatonin (1 nM) for 24 h. Thymidine incorporation was then assessed. Results are mean ± SEM of 4 independent studies, run in quintuplicates, and are expressed in % of control values in the absence of DHT. Bars sharing a common letter do not differ significantly (Student-Newmann-Keul's test).
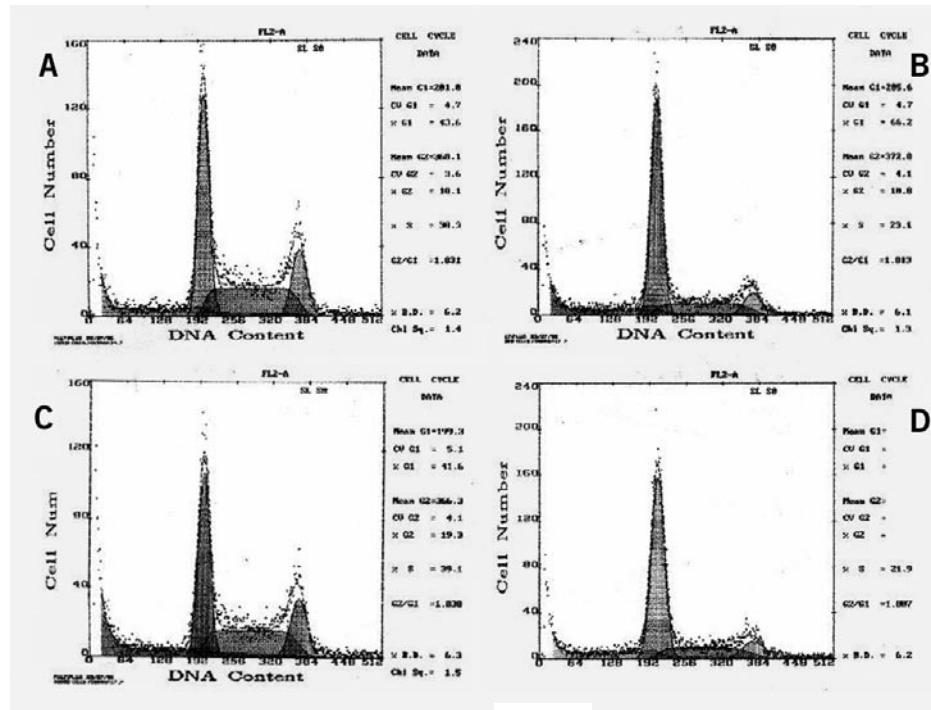
Fig 2: Effects of androgen, melatonin and their combinations on DNA content of PC3-AR cells. Cells were treated with DHT (10^{-12}; 10^{-9} M), melatonin (10^{-9}; 10^{-7} M), their combination or vehicle for 48 h and DNA content of the plates then assessed. Results are mean±SEM of 4 independent studies, run in quintuplicates. Bars sharing a common letter do not differ significantly (Student-Newmann-Keul's test).

tions. At a higher DHT concentration (10^{-9} M) melatonin was less effective.

DNA content: The effects of 48 h incubation of the cells with DHT, melatonin and their combinations on PC3-AR DNA contents are shown in Fig. 2. The androgen (10^{-12} and 10^{-9} M) attenuated DNA content of the culture by 28 and 46%, respectively, as compared to control androgen-free culture. Melatonin (10^{-9}M and 10^{-7} M) attenuated the suppression effected by DHT (10^{-12} M) but was less effective in the presence of higher DHT concentration (10^{-9}M).

Androgen but not melatonin, arrest the cell cycle at the G0/G1 phase

The effects of 24 h incubation of the cells with androgen (10^{-9}M) melatonin (10^{-9}M) and their combinations, on PC3-AR cell cycle phases are shown in Fig. 3. The presence of the S phase was markedly reduced in androgen treated cultures compared to control cultures treated with vehicle (23.1 vs 38.8%, respectively). In parallel %G1 increased (66.6 vs. 43.6% respectively) and % G2 decreased (10.8 vs 18.1% respectively). Melatonin had no significant effect on the cell cycle phases in the absence or presence of DHT and % S phase in the presence and absence of melatonin was 39.1 vs. 38.3, respectively, in

the absence of DHT and 21.9 % vs. 23.1% in its absence. At 10^{-12} M DHT cell cycle phases did not significantly differ from those of control cells, regardless of the presence or absence of melatonin (not shown).

Melatonin does not inhibit binding of the AR or AR-androgen complex to DNA

The interaction of the receptor-androgen complex with target DNA was studied in vitro in PC3-AR and LNCaP cells using an electrophoretic mobility shift assay, which measures the migration velocity of the receptor-DNA probe complex in a non denaturing polyacrylamide gel (Fig. 4). In the LNCaP cells as well as the PC3-AR cells, a receptor-DNA probe complex was found as indicated from the band showing a characteristic retardation of the mobility of the labeled DNA probe (Fig. 4). The specificity of the binding of the labeled probe to the AR is inferred from the absence of retardation of the labeled probe in the presence of excess unlabeled DNA (Fig. 4).

The capacity to form AR-DNA complex was not inhibited and even somewhat enhanced in the melatonin-treated PC3AR as well as LNCaP cells. When normalized per protein content of the sample, enhancement was estimated at ca 30%, for both cell types – consistent with
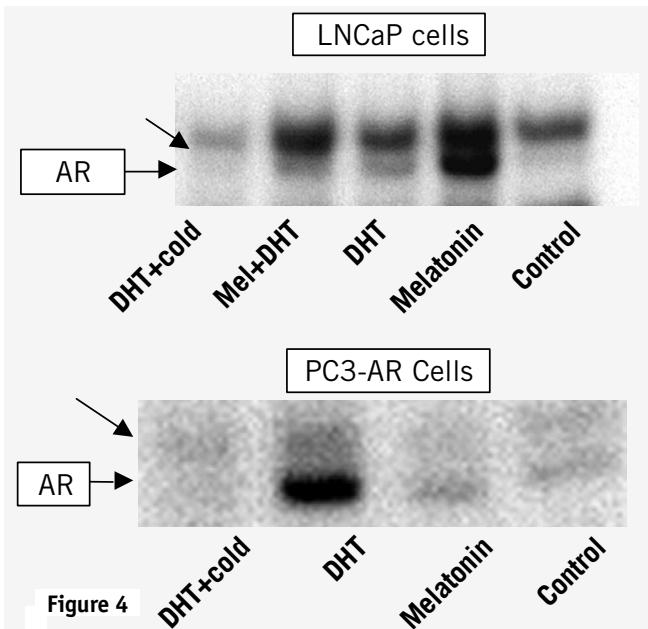
Figure 3

Fig 3: Flow cytometry analysis of PC3-AR cells. Cells (400,000) were treated with either vehicle (**a**), DHT (10^{-9}M, **b)** melatonin (10^{-7} M, **c**) and their combinations (**d**) for 48 h. and then stained with propidium iodide and subjected to flow cytometric analysis. Data recorded in the Figures show the population of nuclei at the G1, S and G2 phases. The results are from a representative experiment out of two independent experiment with similar results.
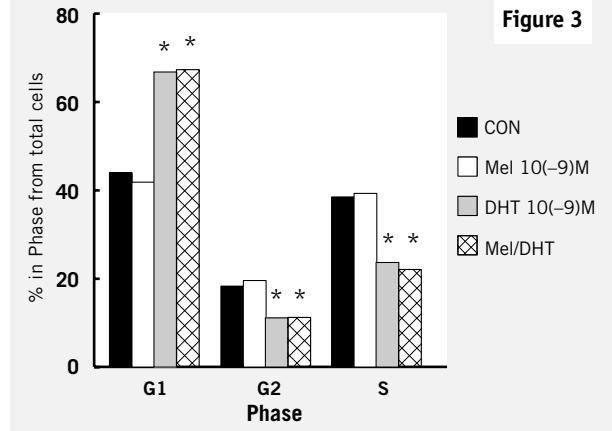
Fig 4: Effects of androgen, melatonin and their combinations on AR binding to DNA AR consensus sequences. LNCaP cells (upper panels) and PC3-AR cells (lower panels) were treated for 1 h with DHT (10^{-9} M) in the absence or presence of melatonin, (10^{-7} M), or 50 fold molar excess of unlabeled AR consensus oligonucleotide (DHT+cold). Representative retardation patterns using whole cell extracts are shown. A horizontal arrow indicates the AR-DNA complex. A tilted arrow indicates the position of the lower migrating complex.

the increase in AR levels in melatonin treated cells [10, unpublished data].

The DNA binding capacity of cell extracts from PC3-AR cells, was markedly enhanced in the presence of DHT; this was also observed in LNCaP cells although to a smaller extent, perhaps due to the lower amount of innate AR expressed in the latter. In addition, another major, slower migrating DNA complex was found regardless of the absence or presence of DHT or melatonin (Fig 4). Notably, the ratio in DNA binding capacity between the AR and this slower migrating band was much higher in LNCaP than PC3-AR cells because the latter express much higher amounts of the AR.

Discussion

The data presented here demonstrate that introduction of AR into PC3 cells results in supersensitivity to androgen-mediated inhibition of growth (evidenced by suppression of thymidine incorporation and DNA content and increase in % cells at the G0/G1 phase at picomolar androgen concentrations. This is perhaps because of the high level of AR expression that might have lead to an increase in association of the AR with unidentified protein(s) responsible for the cell cycle arrest.

Melatonin had some weak androgen-like activities in the cells in the absence of androgen (up-regulated AR-ARE binding, and attenuated cell growth) that may be attributed to the melatonin-mediated increase in AR levels in these as also in LNCaP cells (prostate and unpublished data). Melatonin is not an AR ligand (prostate and unpublished) and therefore its androgen-like activities may not be induced via AR activation.

The data also shows that in the PC3-AR cells, capable of responding to androgen, melatonin partially inhibits DHT effects on thymidine incorporation but not the DHT mediated cell cycle arrest. The attenuation by melatonin of cell growth is thus not due to inducing an accumulation of the cells in G0/G1 phase, suggesting that the attenuating effect is effected across multiple, rather than specific, cell cycle phases.

These anti-androgenic effects of melatonin may not be explained by reduced binding of the AR-androgen complex to the ARE, because no such effect is seen in both transfected wild-type (in PC3-AR) or innate mutant AR (in LNCaP). Moreover, the nuclear exclusion of the AR by melatonin that has been observed in both prostate cell types, are most probably also not derived from a reduced capacity of the AR-androgen complex to bind to the ARE.

This situation differs from that reported in human MCF7 breast cancer cells in which melatonin inhibits estradiol dependent gene expression [14, 15] and modulates transcription of ER [16]. These activities have been associated with inhibition of binding of estradiol-estrogen receptor (ER) complex to its DNA response element (ERE) without decreasing steady state levels of ER or estradiol binding [15]. Notably, as in MCF7 cells [15] melatonin does not effect nuclear exclusion of the innate ER in the PC3AR cells (unpublished data). Therefore, the nuclear exclusion and the effects on binding of the steroid hormone receptors to their respective response elements appear to be independent events induced by melatonin treatment.

Obviously, the fact that melatonin inhibited PC3-AR cell responses to androgen in a selective manner implies that melatonin does not prevent the activation of the AR by androgen but rather some aspects of its activity. Transcriptional regulation by the AR-ARE complex are known to take place alongside interaction of the receptor with basal transcription factors such as the TATA box binding protein or with coactivators that potentiate its activity [17]. In addition, the AR negatively regulates gene expression through interaction with transcription factors such as c-jun to inhibit their DNA binding activity [18], with Ets transcription factors to downregulate their activities [19] and through activation of mitogen-activated protein kinases (MAPKs) [9]. It may thus be suggested that melatonin affects some of these pathways, but not others. Consequently, interference by melatonin results in a diversified response of the cells to androgens rather than a uniform desensitization to androgens.

REFERENCES

1 Gilad E, Laudon M, Matzkin H, Pick E, Sofer M, Braf Z, et al. Functional melatonin receptors in human prostate epithelial cells. Endocrinology 1996; **137**:1412–7.
2 Lupowitz Z, Zisapel N. Hormonal interactions in human prostate tumor LNCaP cells. *J* Steroid Biochem Mol Biol 1999; **68**:83–8.
3 Moretti RM, Marelli MM, Maggi R, Dondi D, Motta M, Limonta P. Antiproliferative action of melatonin on human prostate cancer LNCaP cells. Oncol Rep 2000; **7**:347–51.
4 Xi SC, Tam PC, Brown GM, Pang SF, Shiu SY. Potential involvement of mt1 receptor and attenuated sex steroid-induced calcium influx in the direct anti-proliferative action of melatonin on androgen-responsive LNCaP human prostate cancer cells. J Pineal Res 2000; **29**:172–83.
5 Gilad E, Laufer M, Matzkin H, Zisapel N. Melatonin receptors in PC3 human prostate tumor cells. J Pineal Res 1999; **26**:211–20.
6 Zhou ZX, Wong CI, Sar M, Wilson EM. The androgen receptor: an overview. Recent Prog Horm Res 1994; **49**:249–74.
7 Husmann DA WC, McPhaul MJ, Tilley WD, Wilson JD. Antipeptide antibodies to two distinct regions of the androgen receptor localize the receptor protein to the nuclei of target cells in the rat and human prostate. Endocrinology 1990; **126**:2359–68.
8 Nazareth LV, Stenoien DL, Bingman WE, 3rd, James AJ, Wu C, Zhang Y, et al. A C619Y mutation in the human androgen receptor causes inactivation and mislocalization of the receptor with concomitant sequestration of SRC–1 (steroid receptor coactivator 1) [published erratum appears in Mol Endocrinol 2000; **14**:544]. Mol Endocrinol 1999; **13**:2065–75.
9 Peterziel H, Mink S, Schonert A, Becker M, Klocker H, Cato AC. Rapid signalling by androgen receptor in prostate cancer cells. Oncogene 1999; **18**:6322–9.
10 Rimler A CZ, Levy-Rimler G, Lupowitz Z, Klocker H, Matzkin H, Bartsch G, et al. Melatonin causes nuclear exclusion of the human androgen receptor and attenuates its activity. Cancer Detection and Prevention 2001; **24**(Supplement 1).
11 Culig Z, Stober J, Gast A, Peterziel H, Hobisch A, Radmayr C, et al. Activation of two mutant androgen receptors from human prostatic carcinoma by adrenal androgens and metabolic derivatives of testosterone. Cancer Detect Prev 1996; **20**:68–75.
12 Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991; **139**:271–9.
13 Ikonen T PJ, Kallio PJ, Reinikainen P. Stimulation of androgen regulated transactivation by modulators of protein phosphorylation. Endocrinology 1994; **135**:1359–66.
14 Molis TM, Spriggs LL, Jupiter Y, Hill SM. Melatonin modulation of estrogen-regulated proteins, growth factors, and proto-oncogenes in human breast cancer. J Pineal Res 1995; **18**:93–103.
15 Rato AG, Pedrero JG, Martinez MA, del Rio B, Lazo PS, Ramos S. Melatonin blocks the activation of estrogen receptor for DNA bind-