

Intracellular Signaling Pathways Involved in the Cell Growth Inhibition of Glioma Cells by Melatonin

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Abstract

Melatonin is an indolamine mostly produced in the pineal gland, soluble in water, and highly lipophilic, which allows it to readily cross the blood-brain barrier. Melatonin possesses antioxidant properties and its long-term administration in rodents has not been found to cause noteworthy side effects. In the present work, we found that millimolar concentrations of this indolamine reduced cell growth of C6 glioma cells by 70% after 72 hours of treatment, inhibiting cell progression from G₁ to S phase of the cell cycle. Intraperitoneal administration of 15 mg/kg body weight of melatonin to rats previously injected in the flank with C6 glioma cells reduces tumor growth by 50% 2 weeks after the implant. Inhibition of cell growth does not depend on melatonin membrane receptor activation whereas it seemingly relates to the reduction of intracellular basal free radical levels by 30%. Increase of basal redox state of the cells and constitutive activation of tyrosine kinase receptor [receptor tyrosine kinase (RTK)] pathways, including the extracellular signal-regulated kinase 1/2 (ERK1/2) and the Akt and protein kinase C (PKC) signaling pathways, contribute to the progression of the gliomas leading to the constitutive activation of the redox-dependent survival transcription factor nuclear factor κ B (NF- κ B). The antioxidant effect of melatonin in C6 cells is associated to inhibition of NF- κ B and Akt, but not of ERK1/2. The antiproliferative effect of the indolamine on these cells is partially abolished when co-cubated with the PKC activator 12-*O*-tetradecanoylphorbol-13-acetate, thus indicating that the ability of melatonin to change cellular redox state may be inactivating the pathway RTK/PKC/Akt/NF- κ B. (Cancer Res 2006; 66(2): 1081-8)

Introduction

Besides the advances made in cancer therapy in the past decades, patient survival from malignant gliomas still remains lower than 1 year as chemotherapy is unable to substantially change prognosis (1). This may be due to several causes: low specificity of most chemotherapeutic agents against gliomas, chemoresistance of central nervous system tumors, low tolerance of brain tissue to chemotherapy toxicity, and difficult access of most antitumoral agents to brain tissue. Hematologic toxicity is also dose-limiting even to newest class of chemotherapeutic agents (2).

Melatonin is an indolamine mostly synthesized in the pineal gland although enzymes for its synthesis are widespread. It is

soluble in water and is also highly lipophilic, which allows it to readily cross the blood-brain barrier. Most of the studies on its effects have classically addressed the regulation of circadian rhythms and seasonal reproduction in rodents, which in all cases take place in the nanomolar range (reviewed in ref. 3). Melatonin binds to membrane receptors coupled to several G proteins which activate in the nanomolar range as well (reviewed in ref. 4).

Reports on new properties of melatonin have been made through the last decade. One of the findings that has received most of the attention relates to the antioxidant abilities that could either be directly scavenging free radicals at high (millimolar) concentrations or increasing the activity and expression of antioxidant enzymes at low (nanomolar) concentrations (reviewed in ref. 5). *In vivo* long-term experiments in rodents have proved the lack of any noteworthy side effect and also showed some protection exerted by the indole against several degenerative events occurring with aging, including the incidence of malignant tumors (6, 7).

Melatonin inhibition of cell growth and invasive and metastatic properties in tumoral cell cultures, mainly endocrine tumors, has been previously reported at nanomolar concentrations (8–10). During the last 2 years, also reduction of tumor cell proliferation and induction of cell differentiation effects have been showed using millimolar concentrations of melatonin (11, 12). Furthermore, several reports exist accounting for *in vivo* melatonin decrease of tumor growth (13, 14). Mechanisms involving antitumoral properties of melatonin have been addressed by several groups although no definite intracellular pathway has been described to explain its antineoplastic effects. Estrogen or androgen receptor regulation by melatonin has been shown to be involved in the case of breast or prostate cancer cells, respectively (15, 16); suppression of tumor fatty acid uptake and metabolism was implicated in the protection against hepatoma cancer cell proliferation (13); and the antioxidant effects have been suggested to be involved in the antitumoral effects of millimolar concentrations of melatonin (11, 12).

A previous report described an increase in glioma patient survival by a radioneuroendocrine strategy using radiotherapy plus melatonin compared with radiotherapy alone (17). As far as the authors know, there are no studies on antitumoral action of melatonin on malignant glial tumor cells either *in vitro* or in animal experimental models. In the present work, we show that melatonin, at millimolar concentrations, inhibits glioma cell proliferation as well as *in vivo* tumor growth. We also found that antioxidant properties of melatonin are likely responsible for its antitumoral ability on these cells.

Materials and Methods

Cell culture and reagents. Rat glioma cells (C6) were obtained from ATCC (no. CCL-107) and cultured in DMEM/HAM F-12 mixture supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic

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doi:10.1158/0008-5472.CAN-05-2354

mixture containing 10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin. Cells were maintained at 37°C in a 5% CO₂ atmosphere. For all the experiments, cells were seeded at a density of 20,000/cm². Cell culture reagents were from Sigma Chemical Co. (St. Louis, MO), except for FBS, which was purchased from Invitrogen Life Technologies (Barcelona, Spain). Culture flasks and dishes (Falcon) were acquired from Becton Dickinson BioScience (Le Pont de Claix, France). Melatonin was from Sigma-Aldrich (Milwaukee, WI). Double-stranded oligonucleotides having a nuclear factor κB (NF-κB) and activator protein 1 (AP-1) consensus sequence were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other reagents were purchased from Sigma unless otherwise indicated.

Cell proliferation assays. For the trypan blue exclusion assay, cells were plated on 60-mm plates. After treatments, cells were harvested by trypsinization, stained with trypan blue solution (0.04% w/v), and counted in a NEUBAUER hemocytometer chamber (Sigma-Aldrich, St. Louis, MO). The number of total cells as well as the percentage of viable cells was estimated.

For lactate dehydrogenase (LDH) assay, cells were plated on 24-well plates. After treatments, determination of total and released LDH activity was undertaken following the specifications of *In vitro* Toxicology Assay Kit LDH based Tox-7 (Sigma-Aldrich, St. Louis, MO). Absorbance at 490 nm was determined in an automatic microplate reader (µQuant, Bio-Tek Instruments, Inc., Winooski, VT). Cell viability is represented by ratio between released and total LDH activity and cell number is represented by percentage of total LDH activity versus the control group.

Flow cytometry analysis of cell cycle distribution. For fluorescent-activated cell sorting analysis, C6 cells were seeded in 60-mm plates. After incubation with 1 mmol/L melatonin during 48 hours, cells were harvested by trypsinization. A trypsin inhibitor (0.05%) plus RNase A (100 µg/mL) solution was added afterwards and kept for 10 minutes. Finally, samples were stained with propidium iodide (0.005%) for 10 minutes in the dark. A Beckman Coulter FC500 flow cytometer (Beckton Dickinson) was used for counting 2×10^4 cells per sample. Analysis of cell cycle distribution was carried out using the software ModFit ver 5.2 (Verity Software House, Topsham, ME).

Tumor growth in rats. Rat glioma cells (5×10^6 in 25 µL of DMEM/Ham F-12) were injected s.c. in both dorsal flanks of 20 male Wistar rats of ages 2 months. Ten of them received 15 mg/kg body weight of melatonin s.c. injected daily from day 5 after cell implantation until the end of the experiment. The tumor volume was assessed every 3 days [tumor volume = (long diameter × short diameter²) / 2] for 2 weeks. Animal experiments were done according to the guidelines approved by the Ethical Committee of the National Laboratory Animal Center (Kuopio, Finland) and approved by the local Ethics Committee for Animal Research.

Flow cytometry analysis of intracellular free radicals. Intracellular production of reactive oxygen species (ROS) was evaluated by using the fluorescent probe 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). Cells were seeded in 60-mm plates. After 24 to 48 hours of treatment with 1 mmol/L melatonin, cells were incubated with 10 µmol/L DCFH-DA in serum-free medium during 15 minutes at 37°C in the dark. Afterwards, pellets were resuspended in 500 µL of PBS and 10 µL of a 50 µg/mL propidium iodide solution were added to each tube and incubated for 10 minutes at room temperature in the dark. After shaking, DCF fluorescence of 10,000 alive cells per group (cells without propidium iodide uptake) was measured in a Beckman Coulter FC500 flow cytometer (Beckton Dickinson).

NF-κB and AP-1 DNA-binding assay. To determine NF-κB and AP-1 activation, electrophoretic mobility shift assays (EMSA) were carried out. Nuclear extracts prepared from melatonin-treated cells were obtained following the method described by Dignam et al. (18). Oligonucleotide probes containing the consensus sequence for NF-κB and AP-1 response elements (Santa Cruz Biotechnology) were labeled with [α -³²P]ATP (3,000 Ci/mmol) using T4 polynucleotide kinase 5'-end labeling kit (Amersham Life Science, Buckinghamshire, United Kingdom). Ten micrograms per sample of nuclear extracts were incubated on ice for 30 minutes with 0.4 ng of the labeled oligonucleotide. To reduce nonspecific binding of proteins to the radiolabeled DNA fragment, 1 µg of poly(deoxyinosinic-deoxycytidylic

acid) was added. The specificity of binding was determined by competition with the unlabeled oligonucleotide (100-fold excess). Protein-DNA complexes were resolved on 6% nondenaturing polyacrylamide gels at 250 V for 1.5 hours in Tris-borate EDTA. Gels were dried and exposed to Kodak Biomax X-Ray film.

Intracellular glutathione measurement. Glutathione (GSH) was acid extracted incubating the cells with 6% metaphosphoric acid. For GSH determination, 100 µL of acid extract per sample were incubated for 5 minutes at 25°C with the reaction mixture containing 5 mmol/L EDTA, 0.5 mmol/L 5,5'-dithiobis-(2-nitrobenzoic acid), and 0.4 mmol/L NADPH in 0.1 mmol/L phosphate buffer (pH 7.4). The reaction was started by adding 2 units of glutathione reductase and the formation of 2-nitro-5-thiobenzoic acid was followed by measuring its corresponding benzoate at 412 nm in an automatic microplate reader (µQuant, Bio-Tek Instruments). The total concentration of GSH was determined from a standard curve obtained with known amounts of GSH.

Antioxidant enzyme activities. To measure glutathione peroxidase activity, 100 µg of total proteins per sample were mixed with the reaction mixture containing 1 mmol/L GSH, 250 nmol/L NADPH, and 1 unit of glutathione reductase in 50 mmol/L potassium phosphate. The reaction was started by adding 10 µL of 30 mmol/L *tert*-butylhydroperoxide. NADPH consumption was recorded at 340 nm for 3 minutes in an automatic microplate reader (µQuant, Bio-Tek Instruments). Glutathione peroxidase units were determined from a standard curve carried out with known amounts of glutathione peroxidase.

Superoxide dismutase (SOD) activity was measured using an assay based on the prevention of β-mercaptoethanol-induced NADPH oxidation by SOD activity. Thirty micrograms of total proteins per sample were mixed with the reaction mixture containing 300 nmol/L NADPH, 3 mmol/L EDTA, and 1.5 mmol/L MnCl₂ in TDB buffer until the addition of 20 µL of 10 mmol/L β-mercaptoethanol. NADPH consumption was recorded in a microplate reader (µQuant, Bio-Tek Instruments) at 340 nm for 30 minutes. One enzymatic unit is the amount of enzyme that reduced 50% the β-mercaptoethanol-induced decay in NADPH absorbance at 340 nm.

Western blot. For protein expression analysis, cells were seeded on 100-mm plates. After the treatments, cells were lysed with ice-cold lysis buffer (150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1%v/v Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 µg/mL leupeptin, 10 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L Tris-HCl pH 7.5). Fifty micrograms of total proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Amersham Bioscience). Blots were incubated with appropriate antibodies raised against either the phosphorylated or unphosphorylated form of extracellular signal-regulated kinase [ERK; anti-phospho p42/44 (1:1,000) and anti-p42/44 (1:1,000), Cell Signaling Technology, Beverly, CA] and against the phosphorylated form of Akt, which recognizes all the Akt isoforms [anti-phospho Akt (1:500), BD PharMingen, San Diego, CA], and glyceraldehyde-3-phosphate dehydrogenase [anti-GAPDH (1:100), Santa Cruz Biotechnology]. Immunoreactive polypeptide was visualized using horseradish peroxidase-conjugated secondary antibodies [anti-rabbit immunoglobulin G (IgG) peroxidase conjugated and anti-mouse IgG peroxidase (1:2,000), Calbiochem, San Diego, CA] and enhanced chemiluminescence detection reagents (Amersham Bioscience) following manufacturer-supplied protocols.

Data analysis. Experiments were repeated at least thrice and data were calculated as means ± SE. Statistical comparisons were carried out using one-way ANOVA followed by a Student-Newman-Keuls multiple range test. Statistical significance was accepted when $P < 0.05$.

Results

Inhibition of C6 glioma cells proliferation *in vitro* and *in vivo*. Several concentrations of melatonin were assayed to study its ability in reducing cell proliferation in C6 rat glioma cells. From the concentrations assayed, only 1 mmol/L melatonin reduced cell growth (Fig. 1A). This concentration of the indolamine trimmed C6 growth by 50% at 48 hours and by 70% at 72 hours (Fig. 1B).

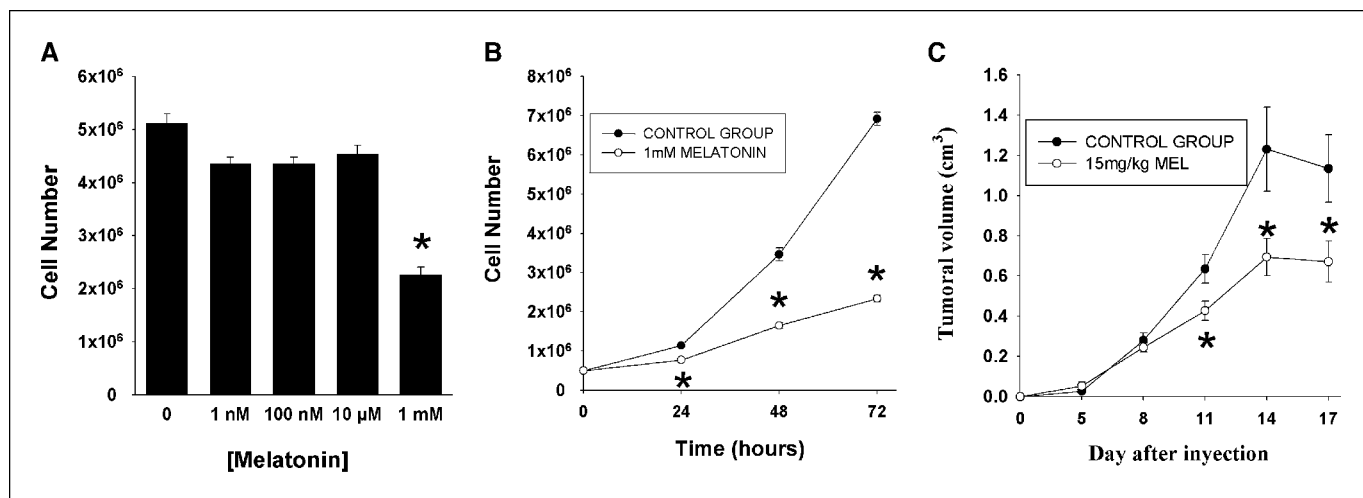


Figure 1. Inhibition of C6 cell proliferation by 1 mmol/L melatonin. **A**, trypan blue cell number count after 48 hours of treatment with several concentrations of melatonin. **B**, cell number after 24, 48, and 72 hours of treatment with 1 mmol/L melatonin. **C**, reduction of tumor growth in Wistar rats. Melatonin (15 mg/kg body weight) was given s.c. daily for 12 days starting at 5 days after C6 injection. *, $P < 0.05$, versus control.

Nanomolar concentrations—most efficient in other cancer cell lines (7)—had no effect under our experimental conditions.

Cell cycle distribution evaluated by flow cytometry showed that treatment of the cells during 48 hours with 1 mmol/L melatonin induces their accumulation in the G_0 - G_1 phase of the cycle (a percentage of $75.61 \pm 0.841\%$ of cells treated with melatonin in G_0 - G_1 versus $69.62 \pm 0.330\%$ of control cells in the same phase). This is associated to a decrease in the number of cells in the S phase ($17.17 \pm 0.790\%$ in the group treated with melatonin versus $20.85 \pm 0.115\%$ in the control cells). The other concentrations of the neurohormone used did not induce any variation in cell cycle distribution (data not shown).

This indicates that 1 mmol/L melatonin inhibits cell progression from G_1 to S phase, thus causing G_1 arrest.

To evaluate the effect of melatonin *in vivo*, C6 glioma cells were implanted in the flank of two groups of Wistar rats. Rats were injected daily with 15 mg/kg body weight of melatonin or the vehicle from the 5th day after the C6 implant. Tumor growth was measured every 3 days and was found to be significantly reduced on the 11th day after the implant as compared with the control group. This reduction was 50% 14 days after the beginning of the experiment (Fig. 1C).

Inhibition of cell proliferation is not mediated by G-coupled membrane melatonin receptors. Melatonin binds to G-coupled pertussis toxin-sensitive membrane receptors. Their activation has been reported to be involved in melatonin antiproliferative properties in human breast cancer (19). To evaluate whether the membrane receptor-coupled signaling cascade was involved in the growth inhibition effects of melatonin in glioma cells, we assayed the effect of several melatonin antagonists. Both the general G-coupled receptor inhibitor pertussis toxin (200 ng/mL) and the membrane melatonin antagonist luzindole (10 μmol/L) failed to prevent the antiproliferative efficacy of melatonin (Fig. 2). This indicates that cell growth arrest induced by this compound in C6 glioma cells is independent of G-coupled receptors.

Melatonin decreases intracellular free radicals. Melatonin has been shown to be an efficient free radical scavenger *in vitro* (5). Furthermore, prevention of induced intracellular increase of free radicals has also been reported for this molecule (20). Evidence of the close relationship between changes in cellular redox state and

its growth has been found (21) and ROS clearly behave as second messengers of extracellular stimuli that trigger cellular signaling pathways (22). Similarly, it has been reported the involvement of ROS in cancer (23) with a neat ROS production by cancer cells (24). To evaluate the possible involvement of the antioxidant effect of melatonin on its antiproliferative properties, we measured intracellular free radicals by flow cytometry. Low concentrations of melatonin (1 nmol/L-10 μmol/L) were not able to decrease DCF fluorescence but 1 mmol/L melatonin decreased by 30% intracellular basal free radical levels (Fig. 3A).

Some other indirect antioxidant properties of melatonin, such as increase of γ -glutamyl synthase expression leading to a total GSH increase (25) and the expression and activity increase of

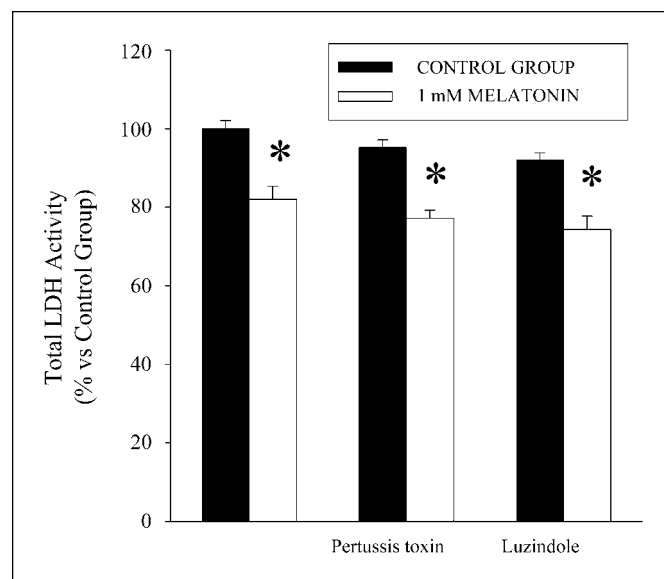


Figure 2. G-coupled melatonin membrane receptor signaling pathways are not involved in antiproliferative effects of melatonin. Coincubation of cells with melatonin and pertussis toxin (200 ng/mL) or with the melatonin antagonist luzindole (10 μmol/L) during 24 hours fails to prevent the inhibition of cell growth by 1 mmol/L melatonin as measured by total LDH activity assay. Pertussis toxin was added to the culture 18 hours before melatonin. Luzindole was added 2 hours before melatonin addition. *, $P < 0.05$, versus control.

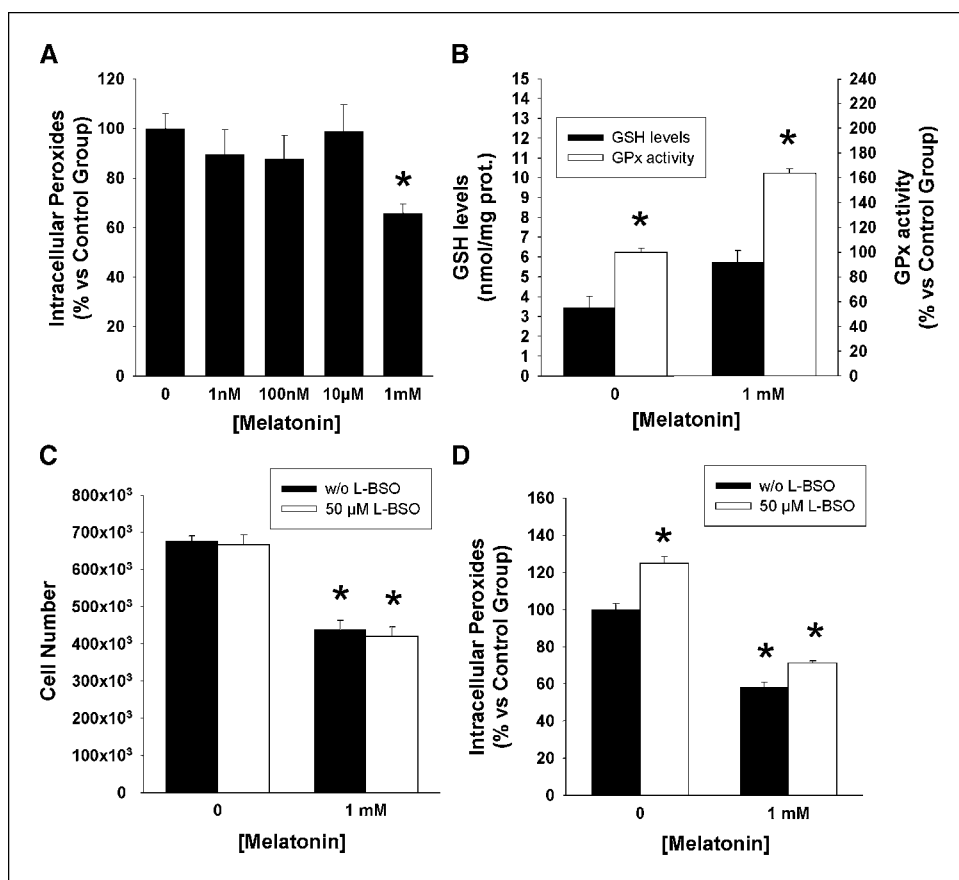


Figure 3. Melatonin decreases intracellular peroxides. *A*, intracellular peroxides after 48 hours treatment with several concentrations of melatonin evaluated by the fluorescent dye DCFH-DA and measuring DCF fluorescence by flow cytometry. *B*, total GSH levels and glutathione peroxidase (GPx) activity after treatment with 1 mmol/L melatonin for 48 hours. *C*, cell number counted by trypan blue after 48 hours of coincubation of the cells with 1 mmol/L melatonin and 50 µmol/L L-BSO. L-BSO was added 2 hours before melatonin supplementation. *D*, intracellular peroxides after treatment during 48 hours with melatonin and L-BSO. L-BSO was added also 2 hours before melatonin addition. *, $P < 0.05$, versus control.

antioxidant enzymes (26, 27), had previously been described. To elucidate if these antioxidant effects of melatonin could be involved in the decrease of intracellular free radicals and the growth inhibition of C6 glioma cells, we measured both total GSH levels and antioxidant enzymes activity. When total GSH levels were analyzed, an increase of a 67% was observed (Fig. 3B). No changes were found in the antioxidant enzyme SOD (data not shown) whereas an increase of 60% in the activity of the antioxidant enzyme glutathione peroxidase was seen (Fig. 3B).

To determine if the increase in GSH, a major intracellular antioxidant, is responsible for the antiproliferative and antioxidant effects of melatonin in C6 cells, buthionine sulfoximine (BSO), an inhibitor of γ -glutamyl cysteine synthetase, the rate limiting enzyme of the GSH synthesis, was given 12 hours before melatonin and maintained in the culture until cells were collected. BSO administration avoided the increase in GSH induced by melatonin (data not shown) but failed to prevent the inhibition of cell proliferation or to decrease free radicals (Fig. 3C and D), indicating that melatonin GSH increase is independent of the intracellular antioxidant and antiproliferative effects. These data also suggest that the increase in the activity of the antioxidant enzyme glutathione peroxidase is not involved in these melatonin effects because it would need GSH as a substrate and the lack of GSH does not affect melatonin actions in C6 cells.

The antioxidant *N*-acetyl cysteine, but not the antioxidant Trolox, reduces intracellular free radicals and inhibits C6 glioma cells proliferation. *N*-Acetyl cysteine (NAC) has been shown to inhibit cell proliferation due to its antioxidant properties (28). To support the hypothesis that melatonin inhibition of cell

growth may be mediated by its antioxidant effects, cells were treated with other known antioxidants, NAC (20 mmol/L) and Trolox (100 µmol/L). NAC inhibited cell proliferation similarly to melatonin, inducing a decrease on the cell number of roughly >60%. On the other hand, Trolox did not inhibit cell growth in C6 glioma cells (Fig. 4A).

When intracellular peroxides were assayed with the fluorescent dye DCFH-DA, we found that NAC decreased free radicals by 40% whereas Trolox failed to show any significant effect (Fig. 4B), thus indicating that, at least for the tested antioxidants, only the one that could reduce C6 cells intracellular free radicals had the ability to decrease their growth.

Melatonin and NAC, but not Trolox, inhibit NF- κ B constitutive activation. Among the several transcription factors regulated by the oxidative status of the cells, the most widely studied are NF- κ B and AP-1. These factors have been reported to be involved in the regulation of major cell processes such as growth, differentiation, and death (29). Melatonin has been shown to regulate NF- κ B and AP-1 activation in other experimental models (25, 30). Given the direct link between cellular oxidative status and activation of these transcription factors and considering the effect of melatonin on basal intracellular free radicals, we evaluated the effect of the indolamine on the activation of NF- κ B and AP-1.

C-6 cells show basal activation of both NF- κ B and AP-1 transcription factors and 1 mmol/L melatonin inhibits NF- κ B activation, showing no effects on the basal activation of AP-1 (Fig. 5A). When the NF- κ B inhibitor parthenolide was used on C6 rat glioma cells, the effects on cell proliferation closely resembled

those obtained with melatonin, producing a decrease in the number of cells without inducing cell death (Fig. 5B).

Studies on the regulation by other antioxidants of the redox-dependent transcription factors mentioned above showed that 20 mmol/L NAC partially inhibits basal activation of NF- κ B whereas the vitamin E analogue Trolox failed to do this (Fig. 5C).

Inhibition of Akt, but not of ERK1/2, is involved in inhibition of cell proliferation by melatonin. Malignant progression of gliomas usually implies activation of receptor tyrosine kinases (RTK) and the intracellular pathways activated by these receptors (Fig. 5D; reviewed in ref. 31). These include mitogen-activated protein kinases (MAPK) and phosphoinositol 3-kinase (PI3K)/Akt pathways. RTKs are also involved in the activation of protein kinase C (PKC) via phospholipase C or PI3K, which has been shown to increase Akt activation (32) and cell proliferation of gliomas (33). All the three kinases, RTKs, MAPK and PKC, may be activated by ROS and inhibited by antioxidants (34–36). To find out if the inhibition of those intracellular

pathways could have any antiproliferative effect in our experimental model, we used inhibitors of ERK1/2 and PKC pathways: PD98059 and calphostin C, respectively. We found that both inhibitors decrease cell growth rate (Fig. 6A) and NF- κ B DNA binding (Fig. 6B) similarly to melatonin. To clarify if PKC inhibition could be involved in the antiproliferative effect of the neurohormone, the cells were coincubated with melatonin and the PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (TPA). We found that activation of PKC by TPA partially prevented the decrease of cell growth induced by melatonin (Fig. 6C). Western blots using the antibodies against the phosphorylated and nonphosphorylated forms of Akt and ERK1/2 show an inhibition of Akt phosphorylation by melatonin whereas no regulation of the MAPK was observed (Fig. 6D).

Discussion

In the present study, we show that the antioxidant indolamine melatonin inhibits growth of C6 rat glioma cells in both cell culture and *in vivo*. We show that this inhibition is associated with a decrease in basal levels of intracellular free radicals and inhibition of the redox-sensitive NF- κ B transcription factor constitutive activation, which in our experimental model seem to be necessary for the said cell growth arrest by antioxidants. Finally, we also show that NF- κ B inhibition is associated with the blocking by melatonin of the Akt constitutive phosphorylation whereas activation of PKC by TPA partially prevents melatonin antiproliferative effect. Taken all together, these data suggest that the antioxidant effect of melatonin, blocking the RTK/Akt/NF- κ B pathway, is playing an important role in the antiproliferative effect of this indolamine in C6 rat glioma cells.

Melatonin possesses membrane receptors coupled to pertussis toxin-sensitive G proteins, previously reported to be involved in the inhibition of cell proliferation of MCF7 breast cancer cells (19). Melatonin membrane receptor K_d is in the nanomolar range, the same concentration shown by Vanecek (4) to be effective in reducing cell growth. Although such low concentrations of melatonin do not have any significant effect on C6 glioma cells, experiments were undertaken to elucidate the possible mediation of its membrane receptors in the antiproliferative effect of high doses of melatonin on these cells. Such experiments included cotreatment of the indole with pertussis toxin as well as the membrane receptor antagonist luzindole. The results indicate that binding to its membrane receptors does not seem to mediate melatonin inhibition of cell proliferation in C6 cells.

Antioxidant properties of this indolamine have also been widely reported (reviewed in ref. 5). The involvement of oxidative stress in tumor promotion and progression has been previously proposed (23) and several types of cancer cells display higher levels of free radicals than normal cells (24). Effects of other antioxidants on tumor cell proliferation have been described before. NAC has been shown to delay the progression from G₀-G₁ to S phase of the cell cycle as well as inhibit tumor cell proliferation (28). The involved mechanism, as suggested by the authors, relates to the ability of NAC to change cellular redox state. We showed that melatonin is able to decrease intracellular free radicals in C6 glioma cells roughly by 30%. Nevertheless, it is not clear how the intracellular redox state could decrease such cell proliferation rate. There are several transcription factors involved in cell growth and death that may be activated by ROS through distinct intracellular pathways (reviewed in ref. 29),

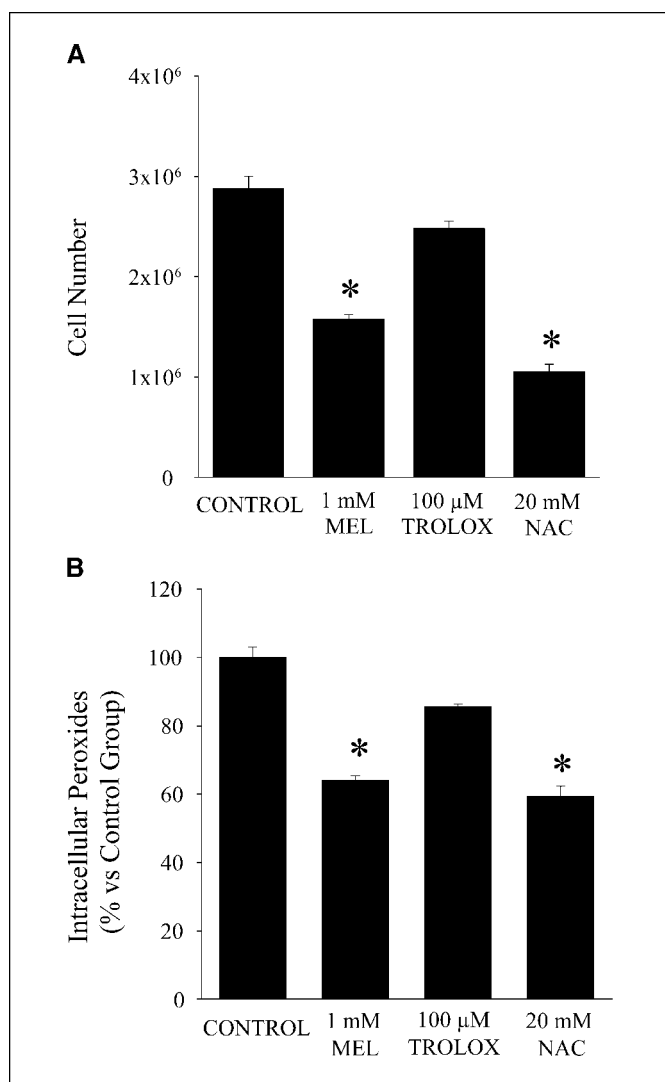


Figure 4. Effect of other antioxidants on C6 cell growth and intracellular peroxides. *A*, comparison of melatonin inhibition of cell growth measured by trypan blue with the effect of NAC and Trolox after 48 hours of treatment. *B*, effect of the treatment during 48 hours with one of the three antioxidants on intracellular peroxides measured by DCF fluorescence in the flow cytometer. *, $P < 0.05$, versus control.

inhibited by antioxidants (37), and previously reported to be constitutively activated in cancer cells (38). Among these, NF- κ B and AP-1 are by far the most widely studied.

NF- κ B activation induces the expression of cell cycle regulators, such as cyclin D1, and promotes transformation (39). This transcription factor is involved in cell growth and tumorigenesis. Inhibition of its constitutive expression in tumoral cells has been shown to decrease cell proliferation (40). Wang et al. (41) reported NF- κ B overexpression and constitutive activation in human diffuse gliomas but not in astrocytes or oligodendrocytes from normal brain or cerebellum. They also found a positive correlation between NF- κ B activation and glioma histologic grade. On the other hand, NF- κ B activation has been linked to chemoresistance whereas inhibition of NF- κ B improves the efficiency of chemotherapy and radiotherapy (42) and new therapeutic strategies to inhibit NF- κ B are being studied. Melatonin has been shown to inhibit tumor cell proliferation although the intracellular pathways involved in the cases studied were rather related to steroid receptors or fatty acid metabolism regulation (8, 13). However, inhibition of NF- κ B by melatonin has been previously reported in other experimental models that show how melatonin prevents NF- κ B activation by oxidative stress (43). In our study, we found that 1 mmol/L melatonin

is not only able to reduce ROS but also inhibits constitutive activation of NF- κ B on C6 glioma cells without showing any effect on the DNA-binding activity of AP-1, the other oxidative stress-regulated transcription factor studied. Inhibition of NF- κ B, on the other hand, seems to be a sufficient condition to inhibit C6 glioma cell proliferation because the NF- κ B inhibitor parthenolide shows the same effect on C6 cell growth than melatonin does. The antioxidant NAC, which also decreased intracellular ROS and cell growth, has been previously reported to inhibit NF- κ B DNA binding activity (44). In the present work, its role in C6 glioma cells has been confirmed. However, Trolox, which does not decrease intracellular peroxides or cell growth in the present experimental model, was also unable to inhibit NF- κ B activation.

Activation of NF- κ B transcription factor may be triggered by several pathways, including the activation of RTKs (31). As a result of the stimulation of these receptors, several intracellular signaling pathways are started, particularly those involving MAPK, PI3K/Akt, and PKC. They all may be additionally phosphorylated directly by ROS (45–47) and are able to activate NF- κ B (Fig. 5D; refs. 44, 48, 49). Our data show that ERK1/2 is not regulated by melatonin in C6 glioma cells, which clearly discards its implication on the neurohormone antiproliferative effects. The fact that an activator of

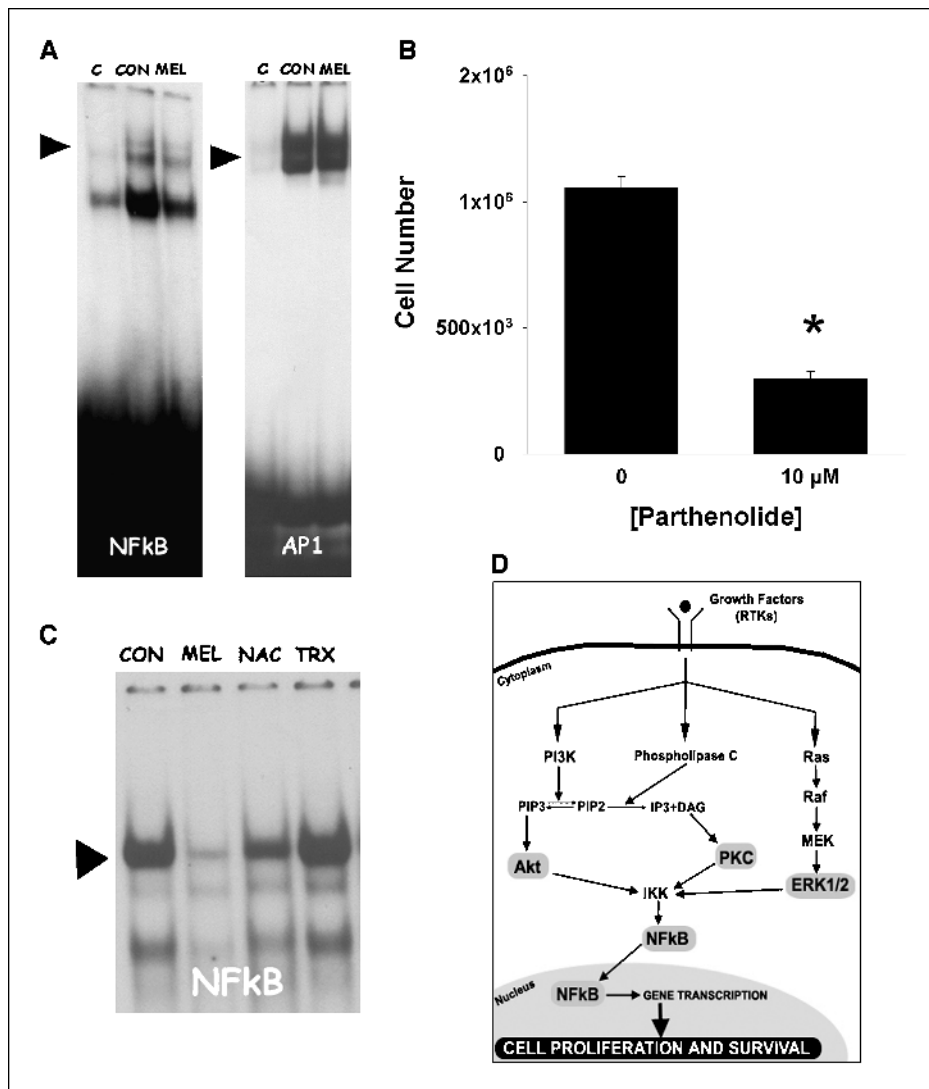


Figure 5. Inhibition of the redox-sensitive transcription factor NF- κ B by melatonin and NAC. *A*, treatment during 48 hours with 1 mmol/L melatonin abolishes NF- κ B, but not AP-1, constitutive activation in C6 glioma cells. DNA binding of the transcription factors was evaluated by EMSA. *B*, inhibition of cell growth by the NF- κ B inhibitor parthenolide after 48 hours of incubation evaluated by trypan blue cell number count. *, $P < 0.05$, versus control. *C*, the antioxidant NAC, but not Trolox, also inhibits NF- κ B DNA binding after treatment during 48 hours. *D*, intracellular pathways that could be implicated in NF- κ B activation in C6 glioma cells. The activity of RTKs induces an increase in NF- κ B activity by modulating MAPK and PI3K/Akt pathways or by activating PKC.

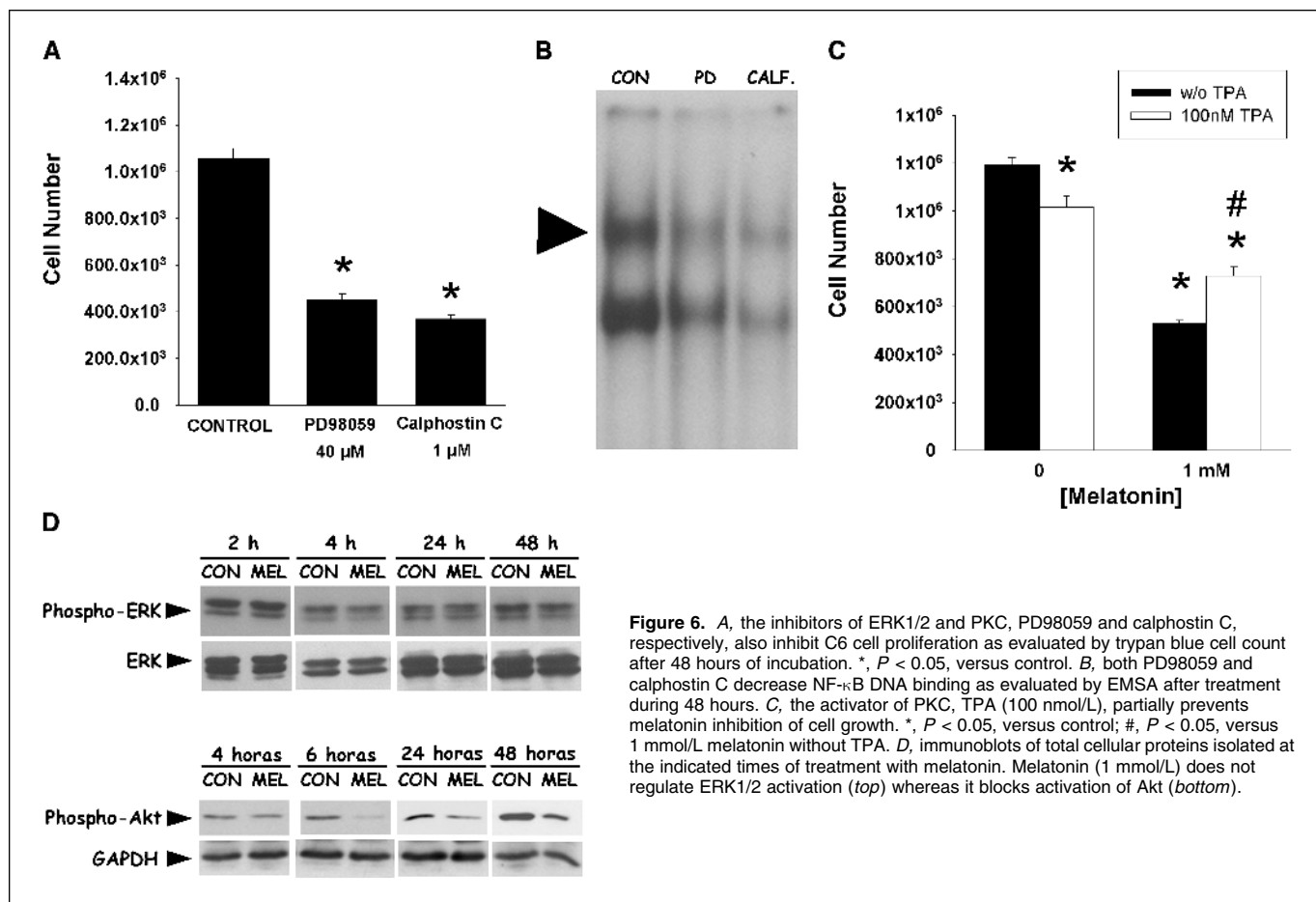


Figure 6. A, the inhibitors of ERK1/2 and PKC, PD98059 and calphostin C, respectively, also inhibit C6 cell proliferation as evaluated by trypan blue cell count after 48 hours of incubation. *, $P < 0.05$, versus control. B, both PD98059 and calphostin C decrease NF- κ B DNA binding as evaluated by EMSA after treatment during 48 hours. C, the activator of PKC, TPA (100 nmol/L), partially prevents melatonin inhibition of cell growth. *, $P < 0.05$, versus control; #, $P < 0.05$, versus 1 mmol/L melatonin without TPA. D, immunoblots of total cellular proteins isolated at the indicated times of treatment with melatonin. Melatonin (1 mmol/L) does not regulate ERK1/2 activation (top) whereas it blocks activation of Akt (bottom).

PKC partially prevents the antiproliferative effect of melatonin suggests that PKC inhibition by this hormone also mediates, at least in part, melatonin antitumoral action. The present results also indicate that Akt phosphorylation is inhibited by melatonin, the Akt pathway being likely involved in the inhibition of glioma cell growth.

Both PKC and Akt induce an increase in NF- κ B DNA binding through phosphorylation of molecules involved in its activation (48, 49). In fact, Akt overexpression in tumor cells has been related to NF- κ B activation (50). In addition, a positive correlation between Akt phosphorylation and NF- κ B activation and between both of them and human glioma grade has been previously reported (41). Bearing in mind that both RTKs and PKC may be directly activated by ROS and inactivated by antioxidants, the antioxidant effect of melatonin could prevent its constitutive activation by the increased ROS in cancer cells and, consequently, prevent Akt phosphorylation and NF- κ B activation, thus decreasing cell proliferation.

In conclusion, only those antioxidants capable of decreasing intracellular free radicals were able to reduce NF- κ B activation and proliferation of C6 glioma cells. In the case of melatonin, both effects may be mediated by inhibition of Akt phosphorylation. The fact that the melatonin effect is partially prevented by a PKC

activator indicates that melatonin, likely through its antioxidant properties, may be avoiding the activation of PKC either directly or by inactivation of RTKs, which in turn would be responsible for the Akt and NF- κ B activation and, consequently, cellular proliferation. Because prevention of the melatonin effect by the PKC activator is only partial, the RTK/PI3K/Akt/NF- κ B direct pathway should be studied in depth.

Acknowledgments

Received 7/6/2005; revised 9/28/2005; accepted 10/20/2005.

Grant support: Fomento de la Investigación Científica y la Tecnología grant PB02-085 and Dirección General de Enseñanza Superior grant SAF2003-03303 (C. Rodríguez); Instituto de Salud Carlos III, Red de Centros de Cancer Red Temática Investigación Cooperativa de Centros de Cáncer grant CO3/10; Fondo de Investigaciones Sanitarias de la Seguridad Social grant PI031596 (I. Antolín); Fondo de Investigaciones Sanitarias grant BEFI 00/9412 (F. Herrera); Fomento de la Investigación Científica y la Tecnología (V. Martín); MAPFRE (J. Rodríguez-Blanco); Fomento de la Investigación Científica y la Tecnología grant BP03-112 (G. García-Santos); and Instituto Universitario de Oncología del Principado de Asturias (P. Carrera-Gonzalez).

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We thank Drs. J.C. Mayo and R.M. Sainz for critical reading of the manuscript.

References

- DeAngelis LM. Brain tumors. *N Engl J Med* 2001;344:114-23.
- Giese A, Westphal. Treatment of malignant gliomas: a problem beyond the margins of resection. *J Cancer Res Clin Oncol* 2001;127:217-25.
- Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. *Endocr Rev* 1991;12:151-80.
- Vanecek J. Cellular mechanisms of melatonin action. *Physiol Rev* 1998;78:687-721.
- Reiter RJ. Oxidative damage in the central nervous system: protection by melatonin. *Prog Neurobiol* 1998;56:359-84.

6. Tamarkin L, Cohen M, Rosell D, Reichert C, Lippman M, Chabner B. Melatonin inhibition and pinealectomy enhancement of dimethylbenz(a)anthracene-induced mammary tumors in the rat. *Cancer Res* 1981;41:4432-6.
7. Pierpaoli W, Regelson W. Pineal control of aging: effect of melatonin and pineal grafting on aging mice. *Proc Natl Acad Sci U S A* 1994;91:787-91.
8. Hill SM, Blask DE. Effects of the pineal hormone melatonin on the proliferation and morphological characteristics of human breast cancer cells (MCF-7) in culture. *Cancer Res* 1988;48:6121-6.
9. Cos S, Fernandez R, Guezmes A, Sanchez-Barcelo EJ. Influence of melatonin on invasive and metastatic properties of MCF-7 human breast cancer cells. *Cancer Res* 1998;58:4383-90.
10. Moretti MM, Limonta P, Maggi R, Motta M, Moretti RM. Growth-inhibitory activity of melatonin on human androgen-independent DU 145 prostate cancer cells. *Prostate* 2000;45:238-44.
11. Sainz RM, Mayo JC, Tan DX, Leon J, Manchester L, Reiter RJ. Melatonin reduces prostate cancer cell growth leading to neuroendocrine differentiation via a receptor and PKA independent mechanism. *Prostate* 2005;63:29-43.
12. Sainz TM, Mayo JC, Tan DX, Lopez-Burillo S, Natarajan M, Reiter RJ. Antioxidant activity of melatonin in Chinese hamster ovarian cells: changes in cellular proliferation and differentiation. *Biochem Biophys Res Commun* 2003;302:625-34.
13. Blask DE, Sauer LA, Dauchy RT, Holowachuk EW, Ruhoff MS, Kopff HS. Melatonin inhibition of cancer growth *in vivo* involves suppression of tumor fatty acid metabolism via melatonin receptor-mediated signal transduction events. *Cancer Res* 1999;59:4693-701.
14. Xi SC, Siu SW, Fong SW, Shiu SY. Inhibition of androgen-sensitive LNCaP prostate cancer growth *in vivo* by melatonin: association of antiproliferative action of the pineal hormone with mt1 receptor protein expression. *Prostate* 2001;46:52-61.
15. Molis TM, Spriggs LL, Hill SM. Modulation of estrogen receptor mRNA expression by melatonin in MCF-7 human breast cancer cells. *Mol Endocrinol* 1994; 8:1681-90.
16. Rimler A, Culig Z, Levy-Rimler G, et al. Melatonin elicits nuclear exclusion of the human androgen receptor and attenuates its activity. *Prostate* 2001;49:145-54.
17. Lissoni P, Meregalli S, Nasetto L, et al. Increased survival time in brain glioblastomas by a radio-neuroendocrine strategy with radiotherapy plus melatonin compared to radiotherapy alone. *Oncology* 1996; 53:43-6.
18. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 1983;11:1475-89.
19. Ram PT, Dai J, Yuan L, et al. Involvement of the mt1 melatonin receptor in human breast cancer. *Cancer Lett* 2002;179:141-50.
20. Garcia-Santos G, Herrera F, Martin V, et al. Antioxidant activity and neuroprotective effects of zolpidem and several synthesis intermediates. *Free Radic Res* 2004; 38:1289-99.
21. Arnold RS, Shi J, Murad E, et al. Hydrogen peroxide mediates the cell growth and transformation caused by the mitogenic oxidase Nox1. *Proc Natl Acad Sci U S A* 2001;98:5550-5.
22. Bae YS, Kang SW, Seo MS, et al. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem* 1997;272:217-21.
23. Hussain SP, Hofseth LJ, Harris CC. Radical causes of cancer. *Nat Rev Cancer* 2003;3:276-85.
24. Sztatowski TP, Nathan CF. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer Res* 1991;51:794-8.
25. Urata Y, Honma S, Goto S, et al. Melatonin induces γ -glutamylcysteine synthetase mediated by activator protein-1 in human vascular endothelial cells. *Free Radic Biol Med* 1999;27:838-47.
26. Antolin I, Rodriguez C, Sainz RM, et al. Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. *FASEB J* 1996;10: 882-90.
27. Martin V, Sainz RM, Antolin I, Mayo JC, Herrera F, Rodriguez C. Several antioxidant pathways are involved in astrocyte protection by melatonin. *J Pineal Res* 2002; 33:204-12.
28. Menon SG, Sarsour EH, Spitz DR, et al. Redox regulation of the G₁ to S phase transition in the mouse embryo fibroblast cell cycle. *Cancer Res* 2003; 63:2109-17.
29. Kamata H, Hirata H. Redox regulation of cellular signalling. *Cell Signal* 1999;11:1-14.
30. Chuang JJ, Mohan N, Meltz ML, Reiter RJ. Effect of melatonin on NF- κ B DNA-binding activity in the rat spleen. *Cell Biol Int* 1996;20:687-92.
31. Kapoor GS, O'Rourke DM. Receptor tyrosine kinase signaling in gliomagenesis. *Cancer Biol Ther* 2003;2: e22-34.
32. Aeder SE, Martin PM, Soh JW, Hussaini IM. PKC- η mediates glioblastoma cell proliferation through the Akt and mTOR signaling pathways. *Oncogene* 2004;23: 9062-9.
33. Begemann M, Kashimawo SA, Choi YA, et al. Inhibition of the growth of glioblastomas by CGP 41251, an inhibitor of protein kinase C, by a phorbol ester tumor promoter. *Clin Cancer Res* 1996;2:1017-30.
34. Sigaud S, Evelson P, Gonzalez-Flecha B. H₂O₂-induced proliferation of primary alveolar epithelial cells is mediated by MAP kinases. *Antioxid Redox Signal* 2005;7:6-13.
35. Boscoboinik D, Szweczyk A, Hensley C, Azzi A. Inhibition of cell proliferation by α -tocopherol. Role of protein kinase C. *J Biol Chem* 1991;266:6188-94.
36. Gopalakrishna R, Chen Zh, Gundimeda U. Modifications of cysteine-rich regions in protein kinase C induced by oxidant tumor promoters and enzyme-specific inhibitors. *Methods Enzymol* 1995;252:132-46.
37. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggrwall BB. Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor- κ B and activated protein-1. *J Biol Chem* 1998;273:13245-54.
38. Rayet B, Gelinac C. Aberrant rel/jfkb genes and activity in human cancer. *Oncogene* 1999;18:6938-47.
39. Karin M, Yamamoto Y, Wang QM. The IKK NF- κ B system: a treasure trove for drug development. *Nat Rev Drug Discov* 2004;3:17-26.
40. Lin A, Karin M. NF- κ B in cancer: a marked target. *Semin Cancer Biol* 2003;13:107-14.
41. Wang H, Wang H, Zhang W, Huang HJ, Liao WS, Fuller GN. Analysis of the activation status of Akt, Nf κ B, Stat3 in human diffuse gliomas. *Lab Invest* 2004; 84:941-51.
42. Mabuchi S, Ohmichi M, Nishio Y, et al. Inhibition of Nf κ B increases the efficacy of cisplatin in *in vitro* and *in vivo* ovarian cancer models. *J Biol Chem* 2004;279: 23477-85.
43. Gilad E, Wong HR, Zingarelli B, et al. Melatonin inhibits expression of the inducible isoform of nitric oxide synthase in murine macrophages: role of inhibition of Nf κ B activation. *FASEB J* 1998;12:685-93.
44. El Bekay R, Alvarez M, Monteseirin J, et al. Oxidative stress is a critical mediator of the angiotensin II signal in human neutrophils: involvement of mitogen-activated protein kinase, calcineurin, and the transcription factor NF- κ B. *Blood* 2003;102:662-71.
45. Kuruganti PA, Wurster RD, Lucchesi PA. Mitogen activated protein kinase activation and oxidant signaling in astrocytoma cells. *J Neurooncol* 2002;56:109-17.
46. Gopalakrishna R, Jaken S. Protein kinase C signaling and oxidative stress. *Free Radic Biol Med* 2000;28: 1349-61.
47. Esposito F, Chirico G, Montesano Gesualdi N, et al. Protein kinase B activation by reactive oxygen species is independent of tyrosine kinase receptor phosphorylation and requires SRC activity. *J Biol Chem* 2003;278: 20828-34.
48. Romashkova JA, Makarov SS. NF- κ B is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 1999;401: 86-90.
49. Lallena MJ, Diaz-Meco MT, Bren G, Paya CV, Moscat J. Activation of I κ B kinase β by protein kinase C isoforms. *Mol Cell Biol* 1999;19:2180-8.
50. Li Y, Sarkar FH. Inhibition of nuclear factor κ B activation in PC3 cells by genistein is mediated via Akt signaling pathway. *Clin Cancer Res* 2002;8:2369-77.