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ORIGINAL ARTICLE

Intracellular redox state as determinant for melatonin antiproliferative vs cytotoxic effects in cancer cells

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Abstract

Melatonin is an endogenous indolamine, classically known as a light/dark regulator. Besides classical functions, melatonin has also showed to have a wide range of antitumoral effects in numerous cancer experimental models. However, no definite mechanism has been described to explain the whole range of antineoplasic effects. Here we describe a dual effect of melatonin on intracellular redox state in relation to its antiproliferative *vs* cytotoxic actions in cancer cells. Thus, inhibition of proliferation correlates with a decrease on intracellular reactive oxygen species (ROS) and increase of antioxidant defences (antioxidant enzymes and intracellular gluthation,GSH levels), while induction of cell death correlates with an increase on intracellular ROS and decrease of antioxidant defences. Moreover, cell death can be prevented by other well-known antioxidants or can be increased by hydrogen peroxide. Thus, tumour cell fate will depend on the ability of melatonin to induce either an antioxidant environment—related to the antiproliferative effect or a prooxidant environment related to the cytotoxic effect.

Keywords: *melatonin, redox state, cancer, proliferation, cell death*

Introduction

Melatonin is a neurohormone mainly synthesized in the pineal gland, although enzymes for its synthesis are widespread. It has been described to regulate circadian rhythms and seasonal reproduction at nanomolar concentration due to its binding to melatonin membrane receptors [1,2]. Reports on new properties of melatonin have been made through the last decades, its antioxidant abilities being the most interesting one [3,4]. Increasing evidences pointed out the role of melatonin in preventing apoptosis in healthy cells. Moreover, *in vivo* long-term experiments in rodents have probed the lack of any noteworthy side effect and also showed some protection against several degenerative events occurring with aging, including the incidence of malignant tumours [5,6]. Thus, melatonin has been described to reduce not only the incidence [7] but also the cancer cell growth [8]. Melatonin inhibition of cell growth, invasiveness and metastasic properties in tumoral cell cultures, mainly endocrine tumours, has been previously reported at nanomolar concentrations [9,10]. However, increasing evidences also showed reduction of cell proliferation and induction of cell differentiation effects using millimolar concentrations [11,12]. Furthermore, several reports exist accounting for *in vivo* melatonin decrease of tumour growth. Additionally, synergy between melatonin and several chemotherapeutic drugs in the induction of apoptosis has also been published [13,14]. Interesting, while melatonin antitumoral properties are generally limited to the inhibition of tumour cell proliferation, during the last years increasing number of reports described an induction of apoptosis by melatonin in several types of cancer

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[15,16], including some haematological cancer cell lines [17,18].

Mechanisms involving antitumoral properties of melatonin have been addressed by several groups, although no widely agreed on pathway has been described to explain its antineoplasic effects. Estrogen or androgen receptor regulation by melatonin has been shown to be involved in the case of breast or prostate cancer cells respectively [19–21]; suppression of tumour fatty acid uptake and metabolism or retinoid-related orphan receptor (ROR) nuclear receptor activation were implicated in the protection against hepatoma or colon cancer cell proliferation [22,23]; and regulation of key intracellular pathways like PI3K/Akt or MAPKs as well as the antioxidant effects have been suggested to be involved in the antitumoral effects of millimolar concentrations of melatonin [12,24].

However, the exact mechanism by which melatonin exerts its antitumoral actions is not fully understood yet. Moreover, no clue has been ever described in order to achieve for differences between melatonin effects on tumour proliferation and cytotoxicity depending on tumour type.

Here we evaluated melatonin antitumoral effects on several tumour types, and we found that cancer cell fate is determined by cellular redox state after melatonin treatment. Thus, melatonin induction of tumour cells death is related to a pro-oxidant environment, increasing intracellular peroxides and decreasing cellular antioxidant defences, while melatonin reduction of tumour cell proliferation is related to an antioxidant environment decreasing intracellular peroxides and increasing cellular antioxidant defences.

Material and methods

Cell culture and reagents

A549 (pulmonary adenocarcinoma), sw-1353 (chondrosarcoma) and A172 (glioblastoma) cell lines were purchased from American Type Culture Collection (Teddington, United Kingdom). Human HL-60 (acute myeloid leukemia: AML) cell line was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). TC-71 (ewing sarcoma) cell line was a generous gift from Dr J.A. Toretsky (Departments of Oncology and Pediatrics, Georgetown University, Washington DC, USA). Cells were maintained at 37°C in a 5% CO_2 atmosphere. Cell culture reagents were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA) except for foetal bovine serum (FBS), which was from Gibco (Invitrogen Life Technologies, Barcelona, Spain). Culture flask and dishes were from Falcon (Becton Dickinson BioScience, Le Pont de Claix, France). Melatonin and all other reagents were purchased from Sigma, unless otherwise indicated.

Evaluation of cell number and cell death

For the MTT assay, cells were plated in 96 well dishes. Treatment was accomplished with 0.2% dimethylsulfoxide (DMSO) (vehicle) or the indicated concentrations of melatonin. Assays were carried out according to the method described by Denizot [25]. Basically, once the treatments were completed, 10 µl of a MTT solution in PBS (5 mg/mL) were added. After 4 hours of incubation at 37°C, one volume of the lysis solution [sodium dodecyl sulphate (SDS) 20% and dimetylformamide pH 4.7, 50%] was added. The mixture was incubated at 37°C overnight, and the samples were measured in an automatic microplate reader (µQuant, Bio-Tek Instruments, Inc., Winooski, VT, USA) at the wavelength of 540 nm.

For the lactate dehydrogenase (LDH) release assay, cells were seeded in 24 well plates. After treatment with melatonin or vehicle for the indicated time, determination of total and released LDH activity was accomplished following specifications of the lactic dehydrogenase based *In Vitro* Toxicology Assay Kit (Sigma). Absorbance was determined using an automatic microplate reader (µQuant; Bio-Tek Instruments, Inc., Winooski, VT, USA) at 490 nm.

Flow cytometry analysis of intracellular free radicals

Intracellular production of peroxides was evaluated by using the fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) following a modification of the method described by Bass et al [26]. When it enters cells, this molecule leads to the fluorescence compound 2′,7′-dichlorodihydrofluorescein upon oxidation by peroxides. Cells were seeded in 6-well plates. Once the treatments were completed, cells were incubated with 10 µM DCFH-DA in serum free medium during 20 min 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 was added to each tube and incubated 10 min at room temperature in dark. DCF fluorescence of 10,000 live cells per group (cells without propidium iodide uptake) was measured in a Beckman Coulter FC500 flow cytometer (Beckton Dickinson).

Fluorescence determination of intracellular glutathione levels

Intracellular gluthation (GSH) levels were evaluated by using the fluorescent probe Thioltracker violetTM (Invitrogen) following manufacture's protocol. When it enters cells, this molecule reacts actively with reduced thiols. Since reduced glutathione represents the majority of intracellular thiols, fluorescence levels

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can be used to estimate intracellular GSH level. Cells were seeded in 96-well plates. Once the treatments were completed, cells were incubated with 10 μM ThiolTrackerTM in serum free medium during 20 min at 37°C in dark. Afterwards, fluorescence of 10,000 cells per group was measured in a fluorescence microplate reader (FLx800; Bio-Tek Instruments, Inc., Winooski, VT, USA) at 405 nm excitation and 525 nm emission.

Real Time Quantitative PCR (Q-RT-PCR)

Total cellular RNA was extracted using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) and reverse transcription were carried out using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Quantitative analysis of mRNA levels was performed by real time PCR using Green PCR Core Reagents (Applied Biosystems) in an HT7900 Real-time PCR system (Applied Biosystems). Each amplification cycle consisted of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C. The Q-PCR was conducted for 40 cycles. One additional step, a melting curve, was added to distinguish specific from nonspecific products and primer-dimers. The melting curve was constructed by increasing the temperature from 60°C to 95°C with a temperature transition rate of 0.2°C/s. The sequences of the sense and antisense primers for human SOD1, SOD2, GPx, CAT and GADPH (housekeeping gene for normalization) are described in the supplementary Table I. Each sample was tested in triplicate, and analysis of relative gene expression data were done using the $2^{-\Delta CT}$ method. To calculate the increases (x-fold) in mRNA levels, the change in cycle threshold (CT) for each gene was calculated by subtracting from their threshold (CT) the corresponding GAPDH CT (internal control). Then ΔCT was calculated by subtracting the average of the CT in the control group from the CT on each experimental group. Changes in gene expression are reported as x-fold increases ($2^{-\Delta CT}$) relative to control group.

Table I (Supplementary). Primers used for quantitative PCR.

Gene	Sequence 5 → 3
SOD1	Forward: 5′-AAGGCCGTGTGCGTGCTGAA-3′
	Reverse: 5′-CAGGTCTCCAACATGCCTCT-3′
SOD 2	Forward: 5′-GCACATTAACGCGCAGATCA-3′
	Reverse: 5′-AGCCTCCAGCAACTCTCCTT-3′
Catalase	Forward: 5′-AAGGTTTGGCCTCACAAGG-3′
	Reverse: 5′-CGGCAATGTTCTCACACAG-3′
Gluthation	Forward: 5′-GTGTATGCCTTCTCGGCGCG-3′
Peroxidase	Reverse: 5′-CGTTGCGACACACCGGAGAC-3′
GAPDH	Forward: 5′-ATGGGGAAGGTGAAGGTCGG-3′
	Reverse: 5′-GACGGTGCCATGGAATTTGC-3′

Statistical analysis

Experiments were repeated at least three times, and data were calculated as mean values ± SE. Statistical analysis were carried out using one-way ANOVA followed by Student-Newman-Keuls test. Statistical significance was accepted when $p < 0.001$.

Results

Melatonin differently regulates cellular redox state depending on tumour type

Melatonin antioxidant activity has been extensively studied showing the ability of the indole to act as a free radical scavenger and a general antioxidant [27]. However, during the last decade a few reports indicate a possible prooxidant activity of melatonin in some types of cancer cells [16,28]. Since our group has previously described melatonin antitumour actions in several tumour types and due to the well-known relation between cancer and cellular redox state, we evaluate melatonin effects on several oxidative stress markers: intracellular ROS production, intracellular gluthation (GSH) levels and antioxidant enzymes expression levels. For that purpose we used a panel of human tumour cells lines (A549-pulmonary adenocarcinoma-; sw-1353-chondrosarcoma-; A172-glioblastoma-; HL-60-acute myeloid leukemia-; and TC-71-Ewing sarcoma-). As showed in Figure 1A, using two different pharmacological concentrations of melatonin, we found that the indolamine induces a time and dose-dependent decrease of intracellular peroxides in sw-1353 chondrosarcoma cells and A172 glioblastoma cells, with a statistic significance after 48 h of 1 mM melatonin treatment, while induces a time and dose dependent increase in TC-71 Ewing sarcoma cells and HL-60 AML cells, starting at 8 h of treatment with both 100 μM and 1 mM melatonin. No significant effects on intracellular peroxides where found at any time or any concentration of melatonin used in A549 pulmonary adenocarcinoma cells. As pointed before, melatonin acts directly as a free radical scavenger, and it indirectly modifies GSH levels and antioxidant enzymes activity and/or expression. While many authors have described increased levels of intracellular GSH after melatonin treatment, a decrease on intracellular GSH levels has been described in correlation to melatonin pro-oxidant activity. In this way, we found that 1 mM melatonin significantly increases GSH levels in A172 and sw-1353 (cells in which we found a decrease of intracellular peroxides) at the same time point, while significantly decreases GSH levels in TC-71 and HL-60 cells (where we found an increase of intracellular peroxides). Differently from intracellular peroxides increase, decrease in GSH levels occurs after 48 h of treatment, probably due to the attempt to eradicate

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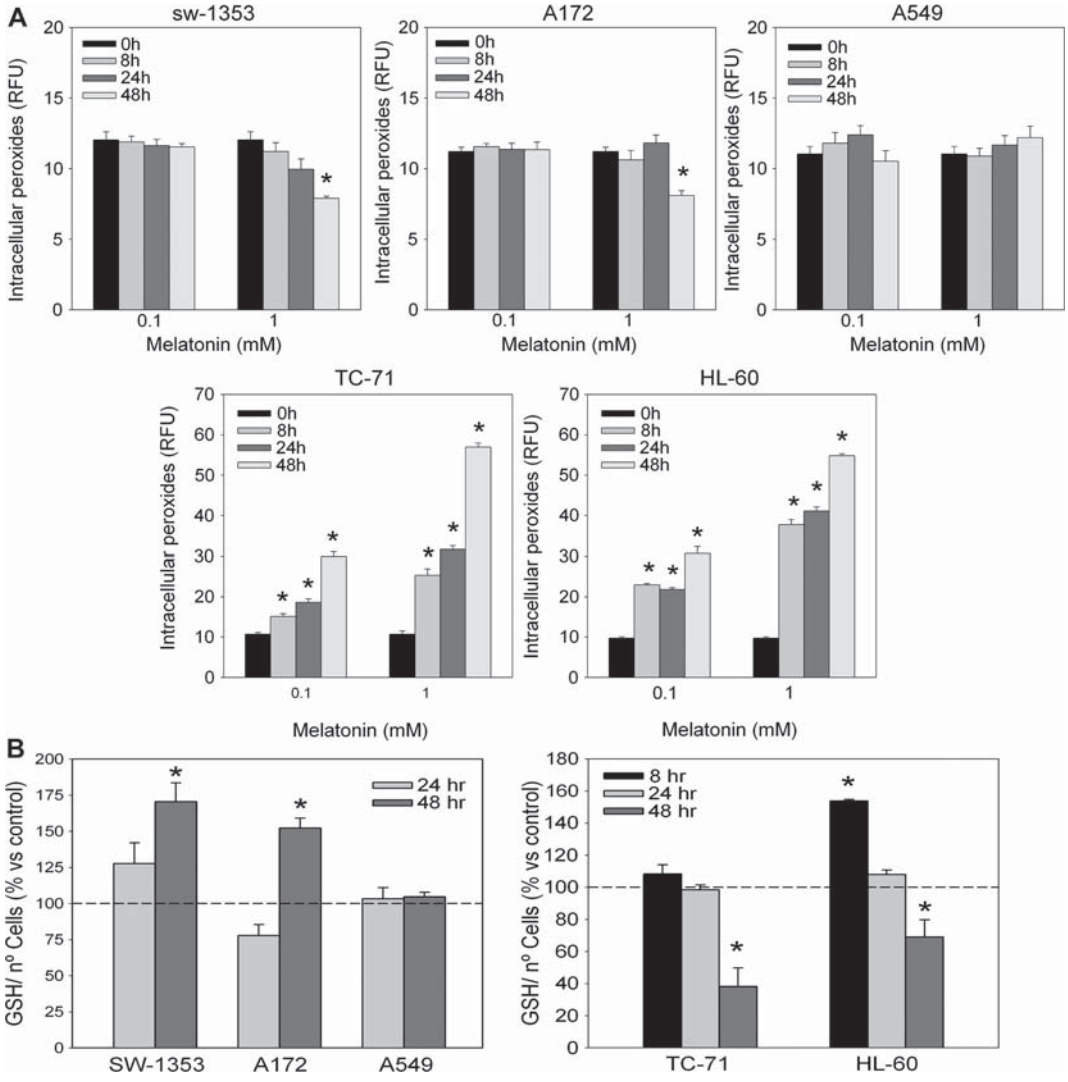

Figure 1. Dual effect of melatonin on cancer cells redox state. A, intracellular peroxides levels after melatonin treatment in several human cancer cell lines (sw-1353, A172, A549, TC-71, HL-60). Cells were treated with or without melatonin (100 μM or 1 mM) for several times (8 h, 24 h, 48 h) and intracellular peroxides levels were measured using the fluorescent probe DCFH-DA. Data are expressed as RFU (relative fluorescence units). $*p \leq 0.05$ vs vehicle-treated cells. B, intracellular glutathione (GSH) levels after melatonin treatment. Cells were treated with or without melatonin (1 mM) for several times (24 h, 48 h for sw-1353, A172, A549 and 8 h, 24 h, 48 h for TC-71, HL-60) and intracellular GSH levels were measured using the fluorescent probe ThiolTracker™. Data are expressed as percentage versus control (vehicle-treated cells: dotted line). $*p \leq 0.05$ vs vehicle-treated cells.

the rapid increase of intracellular peroxides. According to our previous results, no differences were found on intracellular GSH levels in A549 cells in which melatonin does not modify intracellular peroxides (Figure 1B).

Not only GSH levels have been described to be regulated by melatonin but also antioxidant enzymes expression. In agreement to our previous results, we found a divergent response of cancer cells to melatonin. As showed in Figure 2, in those cells where we found a decrease of intracellular peroxides and an increase in intracellular GSH levels (A172 and sw-1353), melatonin induces an increase in mRNA levels as determined by Real-Time PCR of SOD1, SOD2, GPx and catalase after treatment with 1 mM melatonin for 24 h that reverse to basal levels after 48 h. However, in those cells where we found an increase of intracellular peroxides and a decrease in intracellular GSH levels (TC-71 and HL-60), melatonin induces a decrease in mRNA levels for antioxidant enzymes at the beginning (24 h) that reverse to a significant increase in mRNA levels after 48 h treatment. Again, no differences were found after melatonin treatment in A549 cells.

Different effects of melatonin on cellular redox state determine different effects on tumour cells: inhibition of cell proliferation vs cytotoxicity

Reactive oxygen species have been extensively related to cancer responses. On one hand, basal ROS levels have been described to be essential for cancer cell proliferation while higher ROS levels have been described to induce cancer cell death, basically by an

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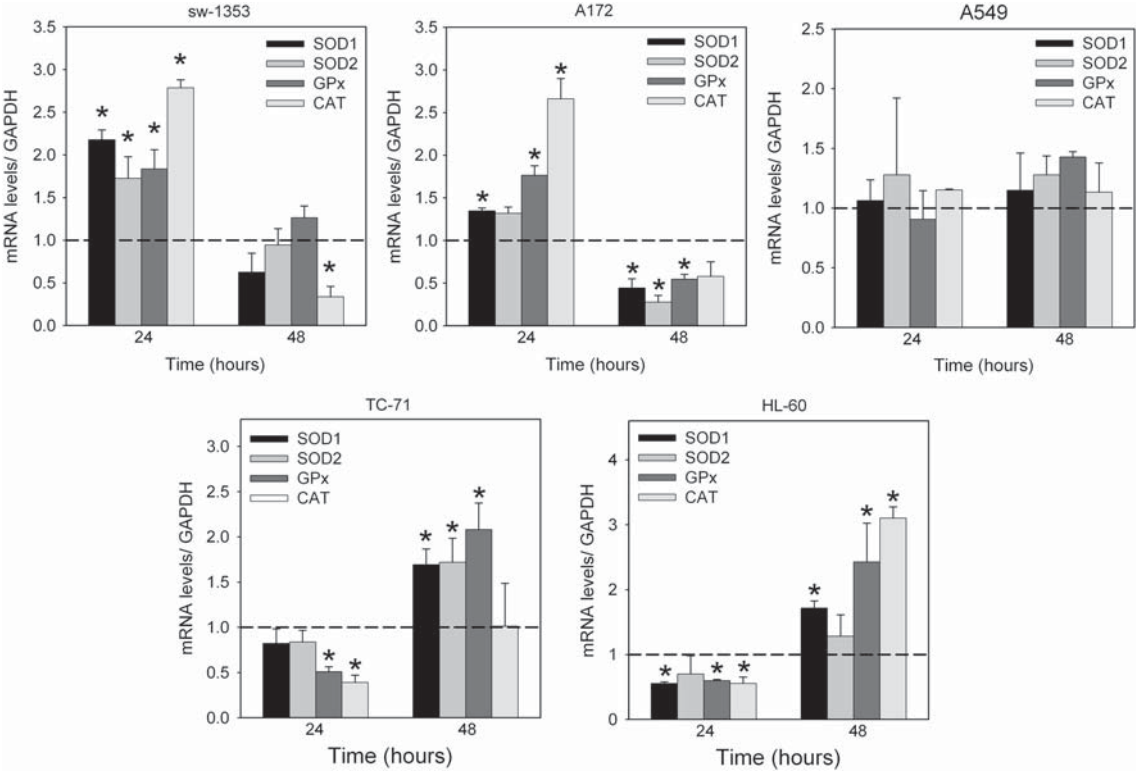

Figure 2. Melatonin differently regulates antioxidant enzymes expression levels. Cells were treated with or without 1 mM melatonin for 24–48 h and SOD1, SOD2, GPx and catalase expression were determined by quantitative PCR assay for mRNA expression levels. GAPDH was used as a house keeping gene. Relative gene expressions are represented as the fold increase compared to basal level (vehicle-treated cells: dotted line). $*p \leq 0.05$ vs vehicle-treated cells.

apoptotic pathway [29]. This way, antioxidant molecules usually decrease cancer cell proliferation while chemorethapy, that usually increases intracellular ROS, induces cancer cell death. Since we have found a dual action of melatonin in cancer cells, we evaluate the response of cancer cells to melatonin treatment in proliferation and cell death. Thus, increasing concentrations of melatonin were assayed for 72 h in our panel of human tumour cells lines. Melatonin induces a dose-dependent decrease in the number of cells in all the five cell lines tested (Figure 3A), however the effect on pulmonary adenocarcinoma cells (A549) was significantly lower than in the rest of the cell lines. Thus, high melatonin concentration (1 mM) only decreases A549 cell number in less than 20% after 72 hr treatment, while the same melatonin concentration decreases the number of cells between 50–70% in the other four cell lines. To test whether the decreased cell number was due to cell death or inhibition of cell proliferation, we next measured LDH release after treatment with 1 mM melatonin for 72 h. Total LDH reflects the number of cells while LDH release reflects cell viability. As shown in Figure 3B, melatonin treatment induces a decrease in total LDH in all cell lines tested except for A549, indicating a decrease in the number of cells. This decrease in cell number corresponds to a decrease in cell proliferation in sw-1353 and A172 cells and an increase in cell death in HL-60 and TC-71 cells as determined by the release of LDH to the extracellular medium (that indicates membrane breaks followed by cell death) (Figure 3C). Although we found a slight increase in LDH release in sw-1353, cells appeared to be healthy as shown in light microscopy images. We confirm by trypan blue exclusion assay that melatonin treatment do not induce cell death in those cells (data not shown). Thus, images from light microscopy (Figure 3D) also show a reduced cell number in sw-1353 and A172 cells while an increased number of dead cells and cellular debris after melatonin treatment in HL-60 and TC71 cells.

In order to evaluate whether the different effect on cell proliferation *versus* cell viability were due to the different effects of melatonin on redox state in our cancer cell lines, we co-incubated melatonin together with other well known antioxidant molecules, trolox and ascorbate. As expected, co-incubation do not prevent antiproliferative effects of melatonin in A172 and sw-1353 cells (Figure 4A). Moreover, it induces a statistically insignificant, slight increase in cell growth inhibition, indicating a relation between inhibition of cell proliferation and melatonin antioxidant effects on those cells. On the other hand, co-incubation decreases melatonin cytotoxicity in TC-71 and HL-60 cells (Figure 4B) indicating that pro-oxidant effects of melatonin are related to cytotoxicity in those cells. Moreover, co-incubation of melatonin together with hydrogen

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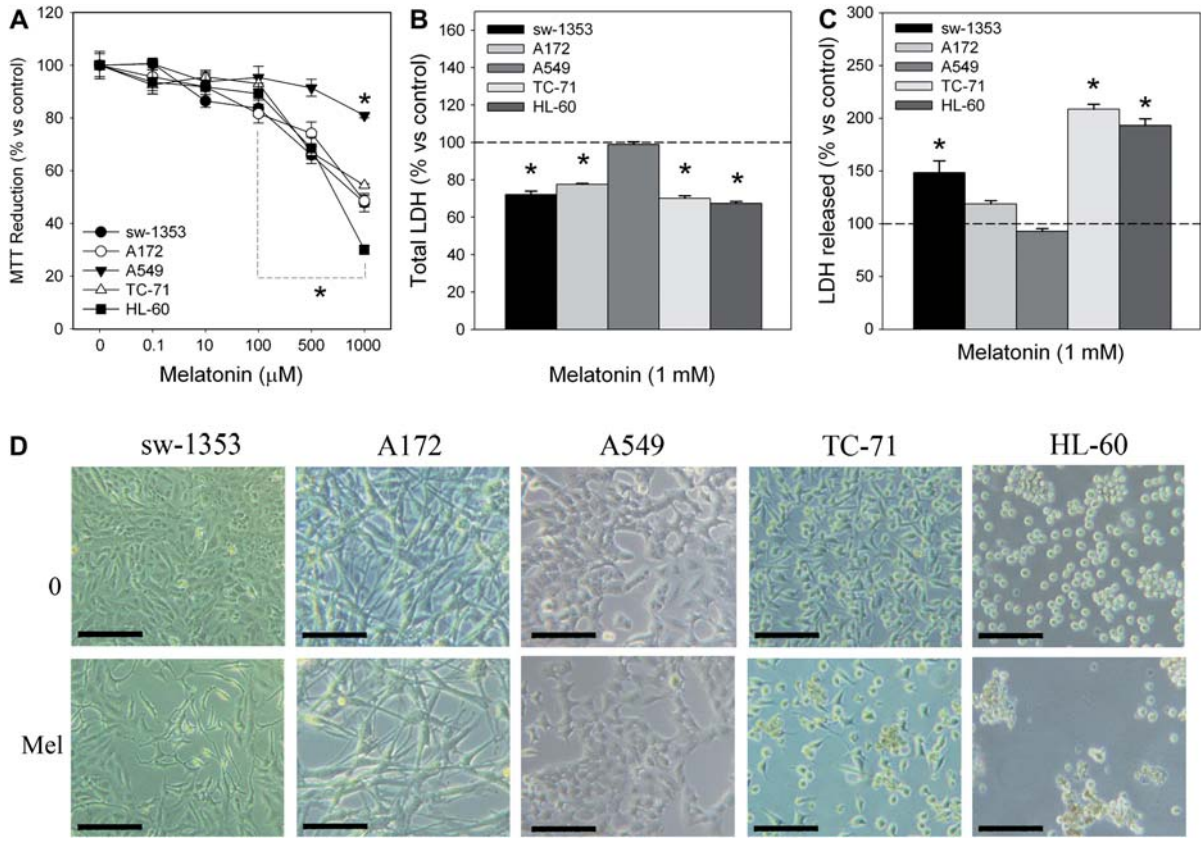

Figure 3. Melatonin cytostatic vs cytotoxic effects on cancer cell lines. A, cells were treated with increasing melatonin concentrations (0.1–1000 μM) for 72 h and cell number was determined by methyl thiazol tetrazolium bromide (MTT) assay. Data are expressed as the percent of control (vehicle-treated cells) values. $*p \leq 0.05$ vs vehicle-treated cells. B, cell proliferation was determined by total lactate dehydrogenase (LDH) values in the same cell lines treated with or without 1 mM melatonin for 72 h. Data are expressed as the percent of control (vehicle-treated cells) values. $*p \leq 0.05$ vs vehicle-treated cells. C, cell death was determined by lactate dehydrogenase (LDH) extracellular release assay. Cells were treated with or without 1 mM melatonin for 72 h and cell death was calculated as the ratio between released and total LDH activity on each experimental group. Data are expressed as the percent of control (vehicle-treated cells) values. $*p \leq 0.05$ vs vehicle-treated cells. D, phase contrast microscopy images showing the decrease in cell number in sw-1353 and A172 cells and the increase of cellular debris in TC-71 and HL-60 cells observed after 72 h of 1 mM melatonin treatment. Bars: 50 μm.

peroxide exerts an additive effect on melatonin cytotoxicity in TC-71 and HL-60 cells (Figure 4C) corroborating the relation between melatonin pro-oxidant effects and cytotoxicity.

Discussion

Results reported in the present paper indicate that the effect of high concentrations of melatonin on tumour activity are related to the ability of the indol to induce either an antioxidant environment – related to the well described antiproliferative effect of melatonin in several tumour types or a prooxidant environment, related to the cytotoxic effects described for the indol in some tumour types –. While melatonin antioxidant properties have been widely reported in the last decades [4], pro-oxidant effects of the indol have been described only in a few recent reports in cancer cells [17].

Even if ROS are implicated predominantly in causing cell damage, relationship between ROS and many important cell functions such as cellular proliferation has been established [30]. Cells can generate endogenous ROS, which are used in the induction and maintenance of signal transduction pathways. In fact, tumoral cells display higher levels of intracellular ROS than normal cells [31,32] and the application of antioxidants usually results in the decrease of cell proliferation [33,34]. Our results demonstrate that in the studied tumour types, where incubation with high concentrations of melatonin induces inhibition of cellular proliferation, this is related to a decrease of intracellular peroxide levels. Since these molecules are involved in tumour progression by activating signal transduction pathways and altering the expression of growth and differentiation-related genes, reduction induced by melatonin could easily modify the growth and proliferation of cancer cells. In this way, direct and indirect antioxidant properties of melatonin have been described both *in vitro* and *in vivo* [35]. Such properties have been reported at both high—micro-millimolar, often named pharmacological, and low—nanomolar, usually called physiological, concentrations. Although high concentrations of melatonin are usually understood as pharmacological, the fact that several body fluids

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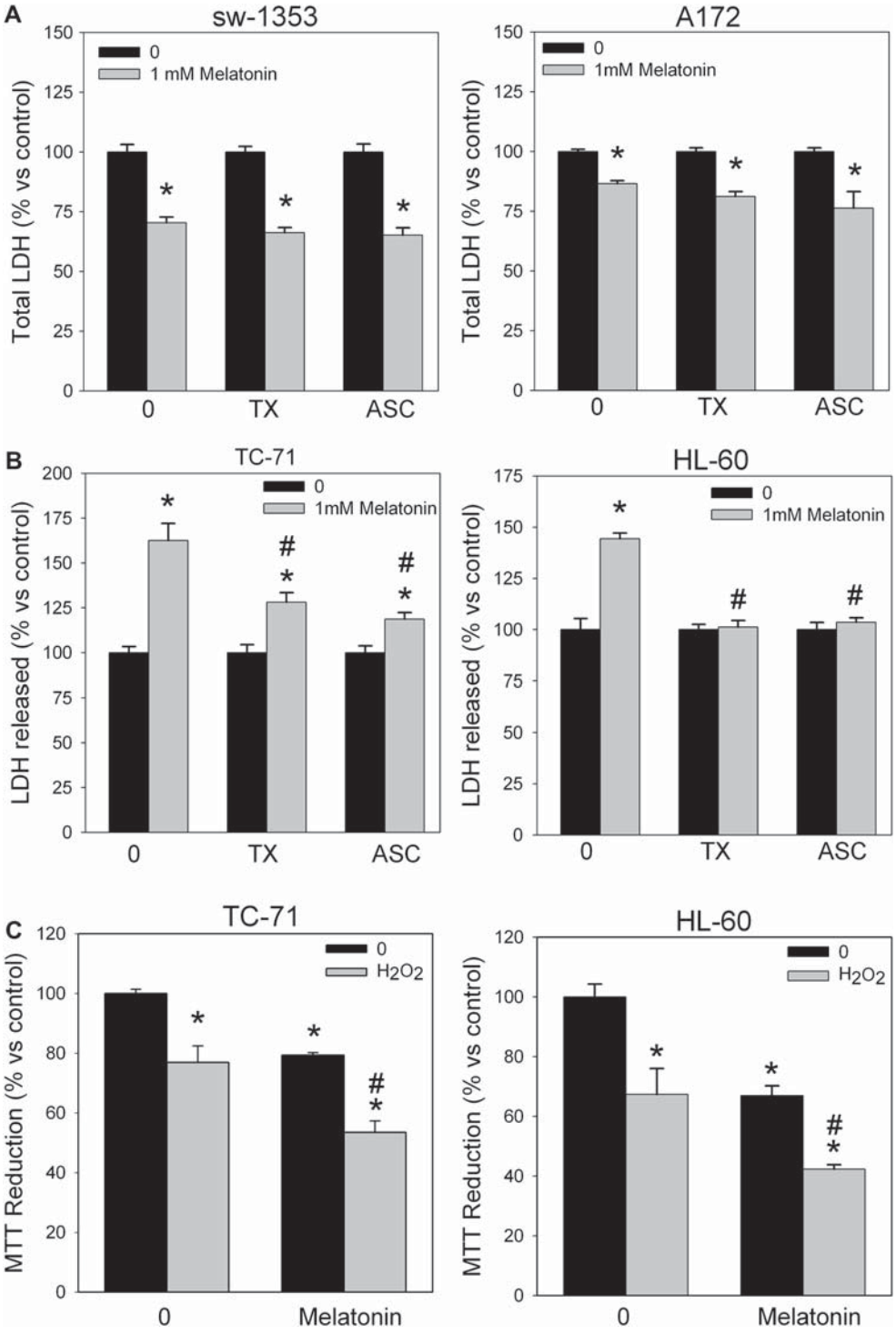

Figure 4. Melatonin antitumour effects are related either to antioxidant or pro-oxidant properties. A, Co-incubation with trolox and ascorbate do not prevent melatonin-induced inhibition of cell proliferation in sw-1353 and A172 cell lines. Cells were incubated with or without melatonin 1 mM, Trolox (TRX) 100 μM or ascorbic acid (ASC) 100 μM or the combination for 72 h. Cell proliferation was determined by total LDH levels. Data are expressed as the percent versus each control group (nonmelatonin treated). $*p \leq 0.05$ vs respective control group. B, Co-incubation with trolox and ascorbate prevents melatonin-induced cell death in TC-71 and HL-60 cell lines. Cells were incubated with or without melatonin 1 mM, Trolox (TRX) 100 μM or ascorbic acid (ASC) 100 μM or the combination for 72 h. Cell death was calculated as the ratio between released and total LDH activity on each experimental group. Data are expressed as the percent versus each control group (non-melatonin treated). $*p \leq 0.05$ vs respective control group. $\#p \leq 0.05$ vs melatonin treated group. C, hydrogen peroxide increases melatonin-induced cell death in TC-71 and HL-60 cell lines. Cells were incubated with or without melatonin 500 μM, hydrogen peroxide (H_2O_2) 10 μM or the combination for 72 h. Cell death was evaluated by methyl thiazol tetrazolium bromide (MTT) assay. Data are expressed as the percent of control (vehicle-treated cells) values. $*p \leq 0.05$ vs vehicle-treated cells. $\#p \leq 0.05$ vs melatonin treated group.

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show melatonin levels several orders of magnitude higher than the nanomolar concentration found in plasma has to be considered. Moreover, melatonin concentrations not only differ in different body fluids but also in different cells and subcellular organelles [36].

Melatonin has previously been reported to present direct free radical scavenging properties at millimolar concentrations [35] and to stimulate the antioxidant defence system mostly at nanomolar concentrations. This system comprises enzymes (SOD1, SOD2, GPx, catalase and so on) and cellular antioxidants, mainly GSH. Regulation of antioxidant enzyme activity and mRNA levels by melatonin has been previously described in both cellular systems as well as animal models, mainly at physiological or near-physiological concentrations of the indol [37]. Moreover, upregulation of intracellular GSH levels has been related to melatonin antitumoral activity in some tumour types after treatment with high concentrations of melatonin [38]. Related to this, our results – showing a decrease in intracellular peroxides after melatonin treatment in the tumour types where this molecule inhibits cell proliferation – correlate well with the increase in cellular antioxidant enzymes expression levels as well as GSH levels.

While ROS function as second messenger molecules is well accepted [30], they are also highly reactive and can cause damage to cellular proteins, lipids and DNA. Therefore, a delicate balance exists between the useful function of these molecules versus their destructive nature. Thus, a biphasic effect has been demonstrated on cellular proliferation with ROS– especially hydrogen peroxide and superoxide – in which low levels induce cell growth but higher concentrations induce cell death [39]. In this way, our results demonstrate that, in other tumour types, high concentrations of melatonin increase intracellular peroxide levels that in turn result in the induction of cell death. According to this observation, few recent reports indicate a possible pro-oxidant activity of melatonin in some types of cancer cells, making them undergo apoptosis [16,28]. In addition, we have found that co-incubation of melatonin together with other well accepted antioxidants at least partially prevents such melatonin induced cell death, while co-incubation together with hydrogen peroxide increases melatonin induced cell death in an additive way, supporting the role of intracellular ROS production in melatonin induced cell death in those tumour types. In this regard, high concentrations of melatonin have been shown not only to induce intracellular ROS production in some tumour types, but also to increase the apoptotic effect of anticancer agents that generate intracellular ROS [13,14]. In contrast to the antiproliferative action, when melatonin acts as a cell death inductor, antioxidant mRNA levels and cellular GSH are decreased, which can easily be related to the increased ROS production. The initial decrease of antioxidant levels is followed by an increase in those levels which can reflect a failure in an attempt to prevent cell death. Taken together, data reported here, open the door to a new question in melatonin antitumoral effects: Which cellular determinants result in a different effect of high concentrations of melatonin on cellular redox status depending on the tumour type and, in turn, in cellular proliferation inhibition *vs* cytotixicity? New studies need to be done in order to clarify this question. The knowledge and understanding of differences between melatonin-sensitive and non-sensitive cells could give us clues to address new therapeutic strategies for particular tumour types resulting in a less toxic therapy.

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Declaration of interest

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