

Melatonin increases the effect of 5-fluorouracil-based chemotherapy in human colorectal adenocarcinoma cells in vitro

Roberto Pariente¹ · Ignacio Bejarano¹ · Ana Beatriz Rodríguez¹ · José Antonio Pariente¹ · Javier Espino¹ 

Received: 15 May 2017 / Accepted: 5 August 2017 / Published online: 17 August 2017
© Springer Science+Business Media, LLC 2017

Abstract Melatonin has antitumor activity via several mechanisms including its anti-proliferative and pro-apoptotic effects. Moreover, it has been proven that melatonin in combination with chemotherapeutic agents enhances chemotherapy-triggered apoptosis in several types of cancer. Therefore, this study was intended to evaluate whether melatonin is able to strengthen the anti-cancer potential of different chemotherapeutic drugs in human colorectal adenocarcinoma HT-29 cells. We found that treatment with 20 μ M cisplatin (CIS) or 1 mM 5-fluorouracil (5-FU) for 72 h induced a decrease in HT-29 cell viability. Furthermore, 1 mM melatonin significantly ($P < 0.05$) increased the cytotoxic effects of 5-FU. Likewise, simultaneous stimulation with 1 mM melatonin and 1 mM 5-FU significantly ($P < 0.05$) enhanced the ratio of cells with an overproduction of intracellular reactive oxygen species and substantially augmented the population of apoptotic cells compared to the treatment with 5-FU alone. Nonetheless, melatonin only displayed moderate chemosensitizing effects in CIS-treated HT-29 cells, as suggested by a slight increment in the fraction of early apoptotic cells that was observed only after 48 h. Consistently, co-stimulation of HT-29 cells with 20 μ M CIS or 1 mM 5-FU in the presence of 1 mM melatonin further increased caspase-3 activation. Apart from this, the cytostatic activity displayed by CIS due to S phase arrest was not affected by concomitant stimulation with

melatonin. Overall, our results indicate that melatonin increases the sensitivity of HT-29 cells to 5-FU treatment and, consequently, the indolamine could be potentially applied to colorectal adenocarcinoma treatment as a potent chemosensitizing agent.

Keywords Melatonin · 5-fluorouracil · Apoptosis · Reactive oxygen species · Colon cancer

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide, accounting for almost 10% of all cancers. According to the estimates, there were nearly 1.4 million new cases of CRC worldwide in 2012, and it is predicted that the number of cases diagnosed annually will rise to 2.4 million by 2035 [1]. Surgical excision is the main treatment, and has proven to be particularly effective in CRC patients diagnosed at early stages (stage I and II). Nonetheless, recurrence after curative resection is a major issue, with recurrence rate being especially high in patients diagnosed at later stages of the disease (stage III and IV) [2]. Adjuvant chemotherapy, which is administered in first- and second-line treatment for advanced CRC, has demonstrated limited efficacy [2]. Therefore, identification of new agents for their potential application in combined therapy for CRC treatment is warranted.

Melatonin, beyond its role as a master regulator of sleep/wake circadian rhythm, is widely accepted as a versatile molecule capable of accomplishing a plethora of physiological functions [3]. Interestingly, melatonin is effective in inhibiting the growth of diverse experimental colon cancer models both in vivo and in vitro [4–6]. Pioneering clinical

Roberto Pariente and Ignacio Bejarano contributed equally to this study.

✉ Javier Espino
jespino@unex.es

¹ Department of Physiology (Neuroimmunophysiology and Chrononutrition Research Group), Faculty of Science, University of Extremadura, 06006 Badajoz, Spain

studies evidenced impairment in melatonin secretion in patients suffering from advanced colorectal tumors [7]. Besides, increased incidence of colorectal cancer has been found in night-shift workers [8], a population characterized by chronic reduction in melatonin production.

Given that the efficacy of chemotherapy is often limited due to tumor resistance, chemotherapeutic drugs are usually utilized together with other anti-cancer agents or antioxidant compounds to increase their tumor-killing potential [9, 10]. In this sense, combination studies of melatonin and chemotherapeutic agents have revealed a potentiating effect of the indolamine on chemotherapy-triggered apoptosis in cancer cells [11]. This held true in cisplatin-treated human ovarian cancer cells [12, 13], doxorubicin-challenged human hepatoma cells [14], cisplatin-stimulated human lung adenocarcinoma cells [15], SK-N-MC human Ewing sarcoma cell line [16], and AR42J rat pancreatic tumor cell line [17], among others. Little is known, however, on the effect of therapeutic synergy of melatonin together with chemotherapeutic drugs in CRC. Hence, the aim of the present study was to investigate whether melatonin may increase the response of human colon adenocarcinoma cells to chemotherapy treatment *in vitro*. Particularly, we explored the anti-cancer effect of combined treatment of cisplatin (CIS) or 5-fluorouracil (5-FU) along with melatonin in the HT29 CRC cell line.

Materials and methods

Reagents

HT29 cell line (ECACC No. 91072201) derived from human colon adenocarcinoma was purchased from The European Collection of Cell Cultures (ECACC) (Dorset, UK). Fetal bovine serum (FBS) and penicillin/streptomycin were acquired from HyClone (Aalst, Belgium). L-Glutamine and Dulbecco's Modified Eagle medium (DMEM) were obtained from Lonza (Basel, Switzerland). Cisdiammineplatinum (II) dichloride (cisplatin), 5-FU, melatonin, HEPES, CHAPS, EDTA, dithiothreitol (DTT), *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (AC-DEVD-AMC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were bought from Sigma Aldrich (Madrid, Spain). Dichlorodihydrofluorescein diacetate (DCFH-DA) was purchased from Life Technologies (Madrid, Spain). Annexin V-FITC apoptosis detection kit was acquired from eBioscience (Barcelona, Spain). Caspase-9 staining kit (Red) was obtained from Abnova (Barcelona, Spain). All other reagents were of analytical grade.

Cell culture and treatment protocol

HT29 cells were grown in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/mL penicillin, and 10 µg/mL streptomycin. Cells were cultured under a humidified atmosphere containing 95% air and 5% CO₂ at 37 °C. Cells were routinely plated into 12-well plates at a density of 1×10^5 cells/mL, unless otherwise indicated, and the viability was >95% in all experiments as assayed by the trypan-blue exclusion method.

Cells were treated with 20 µM CIS, 1 mM 5-FU, or the vehicle, in the absence or presence of 1 mM melatonin for 24 h, unless otherwise specified. Ethanol was used as vehicle and the final ethanol concentration did not exceed 0.1% (v/v). These particular concentrations of drugs were selected because they were previously reported to be effective in inducing cell death [13, 17].

Cell viability assay

Cell viability was evaluated using MTT assay, which is based on the ability of viable cells to convert a water-soluble, yellow tetrazolium salt into a water-insoluble, purple formazan product. Cells were seeded in 12-well plates at a density of 1×10^5 cells/mL, and subsequently, exposed to the appropriate treatment at 37 °C. After the treatments, the medium was removed and MTT was added into each well, and then incubated for 60 min at 37 °C, as previously described [18]. Optical density was measured in an automatic microplate reader (Infinite M200, Tecan Austria GmbH, Groedig, Austria) at a test wavelength of 490 nm and a reference wavelength of 650 nm to nullify the effect of cell debris. Data are presented as percentage above control (untreated samples).

Assay for caspase-3 activity

To determine caspase-3 activity, stimulated or resting cells (1.2×10^6 cells/mL) were sonicated and cell lysates were incubated with 2 mL of substrate solution (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT and 8.25 µM caspase-3 substrate) for 1 h at 37 °C as previously described [19]. The activity of caspase-3 was calculated from the cleavage of the specific fluorogenic substrate (AC-DEVD-AMC). Substrate cleavage was measured with an automatic microplate reader (Infinite M200) with excitation wavelength of 360 nm and emission at 460 nm. Preliminary experiments reported that caspase-3 substrate cleaving was not detected in the presence of the inhibitor of caspase-3, DEVD-CMK. Data were calculated as fluorescence units/mg protein and presented as fold increase over the pre-treatment level (experimental/control).

Detection of reactive oxygen species (ROS) production and caspase-9 activation in double-stained HT29 cells

On one hand, caspase-9 activation was determined using a Caspase-9 Staining Kit (Red). This assay utilizes a caspase-9 inhibitor, LEHD-FMK, conjugated to sulforhodamine (Red-LEHD-FMK) as the fluorescent marker. Red-LEHD-FMK is cell permeable, nontoxic, and irreversibly binds to activated caspase-9 in apoptotic cells. On the other hand, DCFH-DA is a nonfluorescent dye that easily penetrates cell membrane. Once inside the cell, ROS production was analyzed using the probe DCFH-DA, which becomes fluorescent upon oxidation to yield dichlorofluorescein (DCF), fluorescence thus being proportional to ROS generation.

Briefly, stimulated or resting cells were harvested by trypsinization (1.2×10^6 cells/mL), washed twice with PBS, and centrifuged at $500 \times g$ for 5 min; then the supernatant was discarded, and the pellet was resuspended in 400 μ L of PBS containing 0.5 μ L of caspase-9 substrate (Red-LEHD-FMK) and 0.4 μ M DCFH (stock = 20 mM). Cells were incubated for 30 min at 37 °C while the tubes were shaking in the darkness. Then, cells were washed twice, resuspended in 500 μ L of PBS, and immediately analyzed by flow cytometry (MACSQuant VYB; Miltenyi Biotec, Bergisch Gladbach, Germany). Ten thousand events were analyzed using the FL-1 (green; DCF) and FL-3 (red; Red-LEHD-FMK) detectors. Each sample was tested from three to five times in independent experiments. Under all conditions tested, the percentages of DCF⁺/caspase-9⁻ (cells stimulated for ROS production), DCF⁺/caspase-9⁺ (cells entering mitochondrial apoptosis due to ROS overproduction), and DCF⁻/caspase-9⁺ cells (early apoptotic cells) were mainly compared, as previously reported [13].

Determination of apoptosis

Induction of apoptosis was determined using an Annexin V-FITC Apoptosis Detection Kit, according to manufacturer's instructions. Briefly, stimulated or resting cells were harvested by trypsinization (1.2×10^6 cells/mL), washed twice with phosphate buffered saline (PBS), and centrifuged at $500 \times g$ for 5 min; then the supernatant was discarded, and the pellet was resuspended in 200 μ L binding buffer containing 5 μ L of annexin V-FITC. Cells were incubated for 10 min at room temperature, washed twice, and finally resuspended in 200 μ L binding buffer containing 10 μ L of propidium iodide (PI). The cells were immediately analyzed after incubation with the probes by flow cytometry (Cytomics FC500; Beckman Coulter, Vienna, Austria). Ten thousand events were analyzed using

the FL-1 (green; annexin V-FITC) and FL-3 (red; PI) detectors. Each sample was tested from three to five times in independent experiments. Under all conditions tested, the percentages of annexin⁺/PI⁻ (early apoptotic) and annexin⁺/PI⁺ cells (late apoptotic) were mainly compared [20].

Flow cytometry analysis for cell cycle distribution

After treatment, cells (1.2×10^6 cells/mL) were washed with PBS and fixed in 70% ethanol for 30 min at 4 °C. The cells were again rinsed with PBS and resuspended in 500 μ L of PBS containing PI (20 μ M) and RNase A (50 μ M). The samples were kept in the dark at 4 °C for 30 min and then analyzed by flow cytometry (Cytomics FC-500), as described elsewhere [21].

Statistical analysis

Data are presented as mean \pm standard error of mean (S.E.M) for each group. To compare the different treatments, statistical significance was calculated by one-way analysis of variance (ANOVA) followed by post hoc Tukey test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

We explored the impact of co-treatment with melatonin plus CIS or 5-FU on the viability of CRC cells, i.e., HT29 cell line. To do this, MTT assay was performed at different time points (24–72 h). Thus, stimulation of HT29 cells with 1 mM melatonin alone for 24 h resulted in significant ($P < 0.05$) decrease of cell viability (Fig. 1). Increasing the time of exposure (48 or 72 h) to the indoleamine did not further reduce cell viability. As for chemotherapeutic agents, treatment with 20 μ M CIS for 24 h produced a remarkable ($P < 0.05$) drop in cell viability that was far more pronounced after 48 h of treatment (Fig. 1). However, no additional differences were noticed after 72 h of stimulation with CIS. Interestingly, a time-dependent reduction in cell viability was observed when HT29 cells were challenged with 1 mM 5-FU, the maximal effect being achieved after 72 h of treatment (Fig. 1). To examine the possible potentiating effect of melatonin, HT29 cells were incubated with the chemotherapeutic agents in the presence of 1 mM melatonin. The indoleamine proved to be ineffective in further lowering the viability of CIS-treated cells (Fig. 1). On the contrary, melatonin was able to enhance the cytotoxic actions of 5-FU, this effect being statistically significant ($P < 0.05$) after 48 and 72 h of stimulation (Fig. 1).

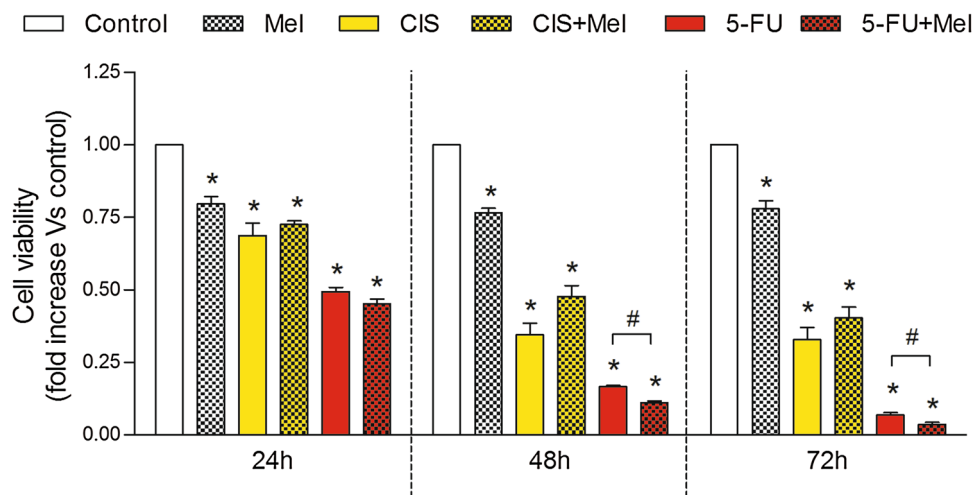


Fig. 1 Melatonin enhances chemotherapy-induced cytotoxicity in HT29 cells. Cells were treated with 20 μ M cisplatin (CIS) or 1 mM 5-fluorouracil (5-FU), or the vehicle (control), in the absence or presence of 1 mM melatonin (Mel) for 24, 48, and 72 h. Cell viability was evaluated by means of the MTT assay. Values are presented as

mean \pm SEM of five independent experiments and expressed as fold increase over control values (untreated samples). * $P < 0.05$ compared to control values. # $P < 0.05$ compared to their corresponding value in the absence of melatonin

To evaluate whether the reduction in cell viability caused by chemotherapeutic agents was connected to apoptotic cell death, the redistribution of phosphatidylserine (PS) in the presence of PI was analyzed. Figure 2a depicts representative cytograms for each treatment assayed. Stimulation of HT29 cells with 1 mM melatonin alone for 24 h produced a slight diminution in the percentage of live cells (annexin⁻/PI⁻) and negligible changes in the amount of both early (annexin⁺/PI⁻) and late (annexin⁺/PI⁺) apoptotic cells (Fig. 2b). Treatments with 20 μ M CIS for 24 h caused a significant ($P < 0.05$) diminution in the percentage of live cells that correlated with subtle increments in the number of both early and late apoptotic cells (Fig. 2b, left panel). Simultaneous administration of CIS and melatonin for 24 h did not sensitize HT29 cells to CIS-induced apoptosis. Moderate chemosensitizing effects, in terms of slightly higher fraction of late apoptotic cells, were observed when CIS plus melatonin treatment was extended up to 48 h (Fig. 2c, left panel). As to 5-FU treatment, when cells were challenged with 1 mM 5-FU for 24 h, the percentage of early and late apoptotic cells was significantly ($P < 0.05$) raised at the expense of the amount of live cells, which was remarkably decreased ($P < 0.05$; Fig. 2b, right panel). Importantly, simultaneous administration of 1 mM 5-FU and 1 mM melatonin for 24 h brought about a further rise ($P < 0.05$) in the number of late apoptotic cells as well as a slight concomitant decrement in the proportion of live cells (Fig. 2b, right panel). This potentiating effect of the indoleamine was even more obvious when cells were treated with 5-FU in the presence of melatonin for 48 h,

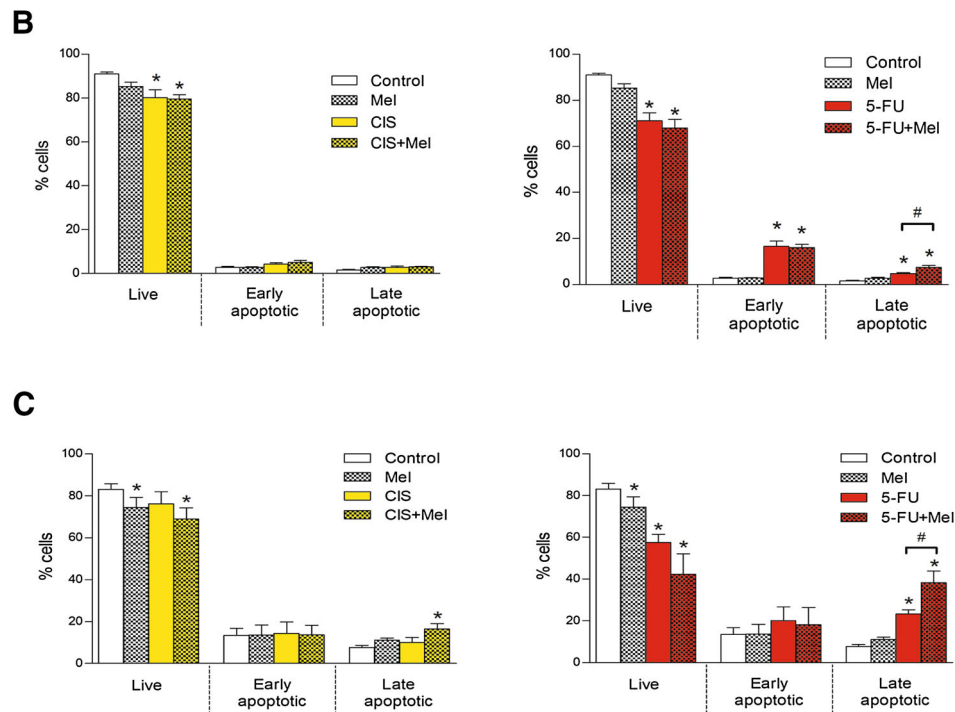
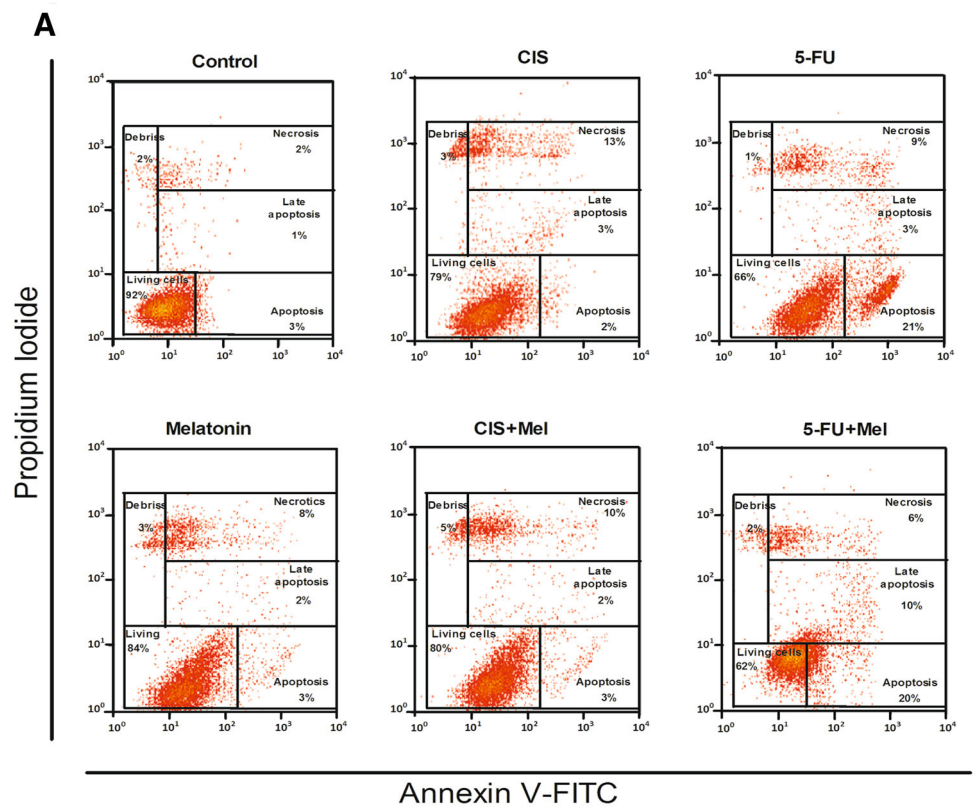
especially in the amount of live and late apoptotic cells ($P < 0.05$; Fig. 2c, right panel).

Given the apoptosis-inducing actions of the chemotherapeutic agents, especially 5-FU, their potential effect on cell cycle progression in CRC cells was subsequently analyzed. Stimulation of HT29 cells with 1 mM melatonin alone for 24 h caused marginal effects on the distribution of the different phases of cell cycle (Fig. 3). On the contrary, treatment with 20 μ M CIS for 24 h led to a significant ($P < 0.05$) accumulation of cells in S phase at the expense of the percentage of cells in G1/G0 and G2/M phases, which was largely reduced ($P < 0.05$; Fig. 3). Likewise, CIS caused a significant ($P < 0.05$) increase in the rate of cells with hypodiploid DNA content (sub-G1 population), i.e., apoptotic cells. Moreover, when HT29 cells were stimulated with 1 mM 5-FU for 24 h, it was observed a noticeable ($P < 0.05$) rise in the number of apoptotic cells and a consequent reduction ($P < 0.05$) in the number of cells in G2/M transition (Fig. 3). Nonetheless, simultaneous administration of melatonin and the chemotherapeutic agents for 24 h did not modify either S phase arrest induced by CIS or rates of sub-G1 population evoked by both CIS and 5-FU (Fig. 3).

Next, we estimated the contribution of intracellular ROS production and caspase-9 activation, a marker for mitochondrial apoptosis, to the chemotherapy-induced cell death. For this purpose, DCF and Red-LEHD-FMK double-stained HT29 cells were analyzed by flow cytometry as described in Materials and methods. Figure 4a depicts representative cytograms for each treatment assayed. Stimulation of HT29 cells with 1 mM melatonin alone for

Fig. 2 The co-treatment with 5-fluorouracil plus melatonin strengthens apoptotic cell death in CRC. HT29 cells were challenged with 20 μ M cisplatin (CIS) or 1 mM 5-fluorouracil (5-FU), or the vehicle (control), in the absence or presence of 1 mM melatonin (Mel) for 24 or 48 h. Apoptotic populations were detected by flow cytometry, as described under “Materials and methods.”

a Representative plots showing the redistribution of phosphatidylserine (annexin V staining) in the presence of propidium iodide (PI) after 24 h of treatment with the indicated combination of drugs. Histograms showing percentages of each cell population after 24 (b) or 48 h (c). Values are presented as mean \pm SEM of seven independent experiments. * $P < 0.05$ compared to control values. # $P < 0.05$ compared to their corresponding value in the absence of melatonin



24 h produced a diminution in the percentage of DCF⁻/caspase-9⁻ (intact) cells, while the proportion of both cells with high ROS levels (DCF⁺/caspase-9⁻) and early apoptotic cells (DCF⁻/caspase-9⁺) slightly raised (Fig. 4). In relation to chemotherapeutic agents, treatments with

20 μ M CIS for 24 h induced an increase in the amount of cells entering mitochondrial apoptosis due to ROS overproduction (DCF⁺/caspase-9⁺) and early apoptotic cells ($P < 0.05$) at the expense of the percentage of intact cells, which was reduced (Fig. 4b, left panel). Co-treatment with

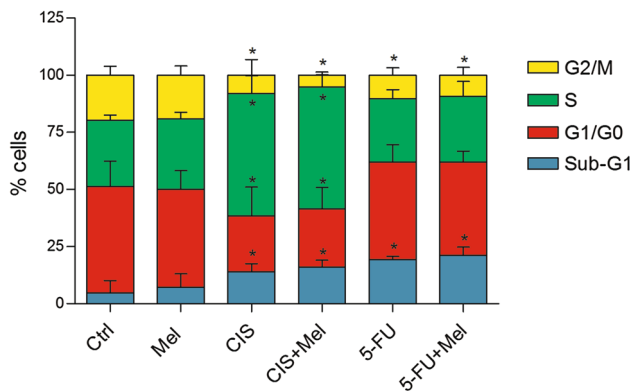


Fig. 3 Cisplatin and 5-fluorouracil differently affect cell cycle distribution. HT29 cells were stimulated with 20 μ M cisplatin (CIS) or 1 mM 5-fluorouracil (5-FU), or the vehicle (control), in the absence or presence of 1 mM melatonin (Mel) for 24 h. Cell cycle distribution was determined by flow cytometry analysis using ethanol-fixed, propidium iodide-stained cells. Data of cell cycle distribution were summarized and presented as percentage of cells. Rate of cells with hypodiploid DNA content (sub-G1 population) were considered apoptotic. Values are presented as mean \pm SEM of six separate experiments. * $P < 0.05$ compared to its corresponding control value

CIS plus melatonin did not strengthen pro-oxidant and pro-apoptotic effects of CIS against HT29 cells (Fig. 4b, left panel). As for 5-FU, stimulation of HT29 cells with 1 mM 5-FU for 24 h remarkably ($P < 0.05$) increased the number of cells with high ROS levels and the proportion of cells entering mitochondrial apoptosis due to ROS overproduction while dramatically reducing the amount of intact cells (Fig. 4b, right panel). Interestingly, the pro-apoptotic effects of 5-FU were potentiated when 1 mM 5-FU and 1 mM melatonin were simultaneously administered for 24 h. In fact, the number of early apoptotic cells was significantly ($P < 0.05$) improved and the percentage of intact cells was concomitantly lessened (Fig. 4b, right panel) compared to HT29 cells treated with 5-FU alone.

Finally, we analyzed activation of caspase-3, a key downstream effector of apoptosis, in chemotherapy-treated HT29 cells. Treatment of cells with 1 mM melatonin alone for 24 h gave rise to a large rise in caspase-3 activity ($P < 0.05$; Fig. 5). Moreover, treatments with 20 μ M CIS and 1 mM 5-FU for 24 h also led to enhanced caspase-3 activity ($P < 0.05$; Fig. 5). It is worth mentioning that melatonin was able to enlarge chemotherapy-evoked caspase-3 activation. In fact, treatment of HT29 cells with 20 μ M CIS or 1 mM 5-FU in the presence of 1 mM melatonin for 24 h markedly ($P < 0.05$) potentiated caspase-3 activation.

Discussion

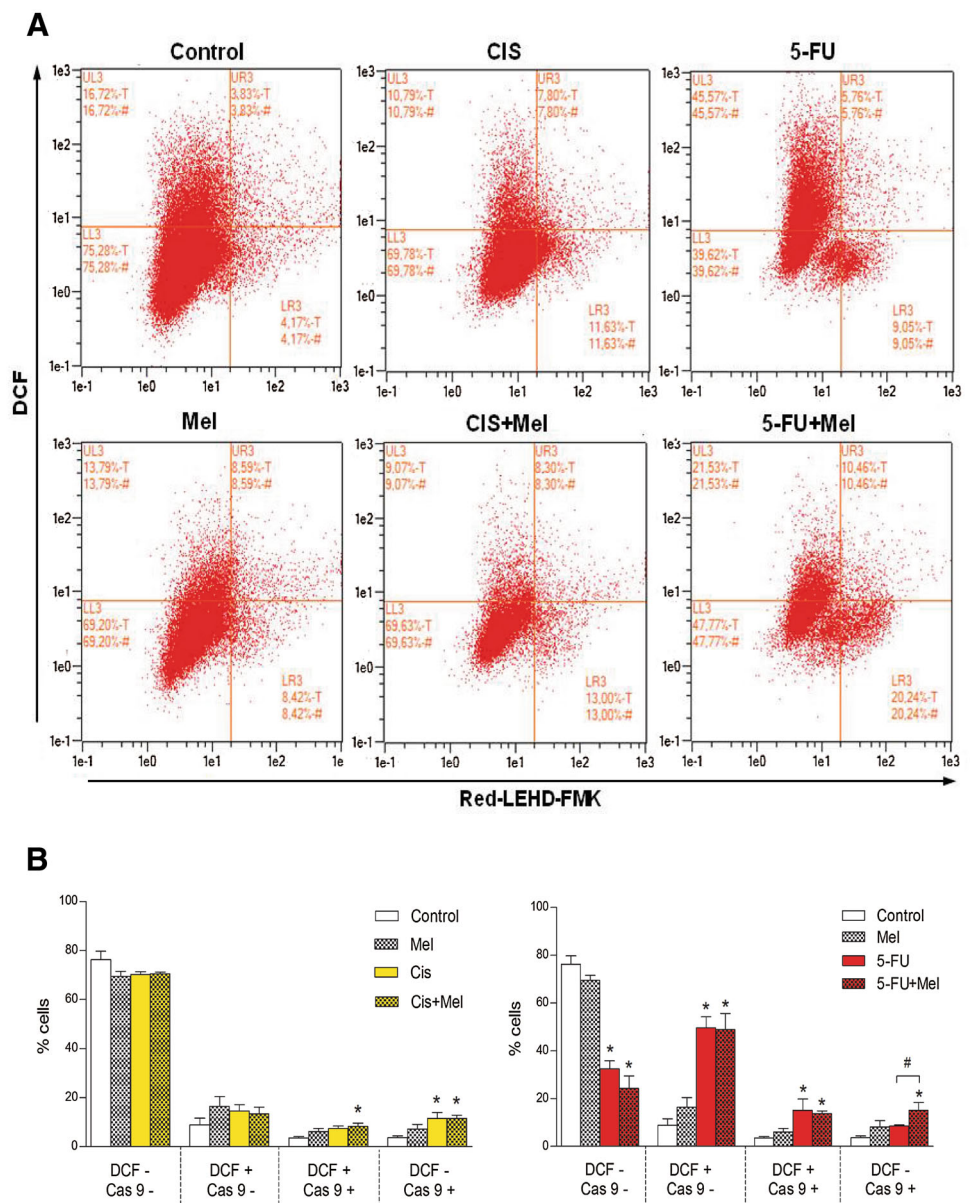
Induction of cell death and inhibition of cell survival pathways are the main principles of cancer therapy. So far, 5-FU remains the single most effective and commonly used

chemotherapeutic agent for the treatment of CRC. Administration of 5-FU, which irreversibly inhibits thymidylate synthetase, causes shortage of deoxythymidine monophosphate and subsequent cell death in rapidly dividing cancerous cells [22]. Nevertheless, 5-FU treatment results in limited therapeutic efficacy owing to undesirable side effects in normal cells and development of tumor resistance [23]. Recent studies have demonstrated that combined chemotherapy is useful for overcoming drug resistance [24] and, therefore, concurrent use of 5-FU and other agents would provide a more efficient approach to sensitize CRC cells to chemotherapy. In this context, the use of melatonin as adjuvant therapy could be of great interest, as previously suggested [25].

In the current study, we examined the anti-cancer potential of co-treatment of melatonin together with CIS or 5-FU on human CRC cells. Melatonin per se manifested mild cytotoxic, pro-oxidant, and pro-apoptotic effects towards HT29 cells. Similar results have been previously shown by our group [18, 26] and others [27, 28] in different cancer cell lines. Interestingly, the indoleamine further strengthened the cell killing capacity of 5-FU, but did not significantly sensitize CRC cells to CIS challenge nor did it modify CIS-driven cell cycle arrest. These diverse findings can be explained on the basis of the different mechanisms driving the anti-cancer effects of CIS and 5-FU. Indeed, CIS predominantly depicted oncostatic actions due to S phase arrest (Fig. 3) that led to a reduction in cell proliferation over time (Fig. 1), whereas 5-FU noticeably triggered ROS-dependent apoptosis (Figs. 2, 4b) that in turn resulted in a drastic drop in cell viability (Fig. 1). It is also worth highlighting that, although there is certain consensus that melatonin is capable of increasing the efficacy of chemotherapeutics [12–17], some previous studies have also yielded contradictory results. In fact, it has been described that melatonin hampers idarubicin-induced nuclear fragmentation in leukemic K562 cells [29] and attenuates CIS anti-cancer actions in liver carcinoma HepG2 cells [30]. Other studies have also reported that combination of melatonin with chemotherapeutic agents such as cytarabine and etoposide does not achieve extra benefits in anti-cancer treatment [13, 31]. Even so, a substantial number of studies provides evidence that melatonin mitigates side effects produced by chemotherapeutic agents [32–34]. Particularly, it is suggested that the indoleamine, which renders protection against mitochondrial oxidation [19], might alleviate oxidative-based chemotherapy-induced side effects [32–34].

Melatonin is a well-known, powerful antioxidant [35] that has been avowed as apoptosis inducer in various types of cancer, including leukemia, cervical, and colon cancers [13, 26, 36–38]. Besides, melatonin has been reported to overcome drug resistance in some cancers [39]. Herein, we

Fig. 4 Melatonin synergizes caspase-mediated apoptosis induced by chemotherapeutic agents. HT29 cells were treated with 20 μ M cisplatin (CIS) (a) or 1 mM 5-fluorouracil (5-FU) (b), or the vehicle (control), in the absence or presence of 1 mM melatonin (Mel) for 24 h. ROS production and caspase-9 activation were analyzed in double-stained cells (DCF and Red-LEHD-FMK) by flow cytometry, as described under “Materials and methods.” **a**, **b** Histograms show percentages of each cell population. Values are presented as mean \pm SEM of six independent experiments. * $P < 0.05$ compared to control values. # $P < 0.05$ compared to their corresponding value in the absence of melatonin



have provided evidence that melatonin is able to enhance the cytotoxic activity of 5-FU in CRC cells, which correlated with the induction of apoptosis, as ascertained by annexin V assays. In fact, when compared to 5-FU alone, simultaneous administration of 5-FU and melatonin brought about a meaningful rise in the proportion of apoptotic cells and a parallel reduction in the population of live cells (Fig. 2b). Apoptosis or programmed cell death is a fundamental physiological process that plays a major role in tissue homeostasis, organ development, and elimination of defective or potentially dangerous cells [40]. Traditionally, two general apoptotic pathways have been described: extrinsic and intrinsic pathways. The latter, also known as mitochondrial pathway, is mediated by mitochondrial alterations. Thus, in response to apoptotic

stimuli, several proteins are released from the intermembrane space of mitochondria into the cytoplasm. Among them, cytochrome *c* is a key protein that mediates the activation of caspase-9, an initiator caspase that triggers the activation of effector caspases, including caspase-3, which leads to the loss of cellular structure and function, and ultimately results in cell death [41]. In this study, we have also demonstrated that melatonin remarkably improved 5-FU-mediated activation of caspase-9 (Fig. 4b) and caspase-3 (Fig. 5). Consequently, our findings indicated that the inhibition of cell proliferation caused by the concomitant administration of 5-FU and melatonin is associated with the enlarged activation of the caspase-9-dependent mitochondrial apoptotic pathway. Nonetheless, melatonin potentiating effects were apparently independent

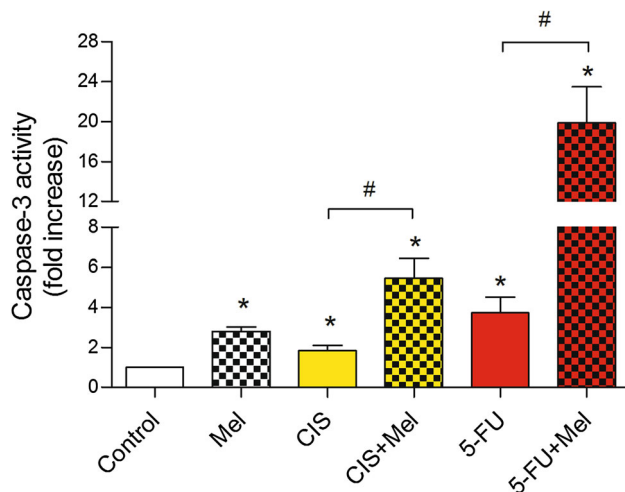


Fig. 5 Melatonin enhances caspase-3 activation induced by chemotherapeutic agents. HT29 cells were treated with 20 μ M cisplatin (CIS) or 1 mM 5-fluorouracil (5-FU), or the vehicle (control), in the absence or presence of 1 mM melatonin (Mel) for 24 h, and then caspase-3 enzymatic activity was estimated as described under “Materials and methods.” Values are presented as mean \pm SEM of eight independent experiments and expressed as fold increase over the pre-treatment level (experimental/control). * $P < 0.05$ compared to control values. # $P < 0.05$ compared to their corresponding value in the absence of melatonin

of 5-FU-evoked ROS generation, as judged by the same proportion of cells entering mitochondrial apoptosis due to ROS overproduction in the absence and presence of melatonin (Fig. 4b).

In general, our findings fit into previous recent data showing that the enhanced anti-cancer effect of melatonin and 5-FU in CRC cells was mediated through modulation of caspase-dependent apoptosis [42]. Likewise, the same authors also indicated that melatonin synergized the anti-tumor effect of 5-FU by means of other mechanisms, i.e., by suppressing the phosphoinositide 3-kinase (PI3K)/AKT survival pathway and by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-dependent inducible nitric oxide synthase (iNOS) signaling [42]. The contribution of these two alternative pathways to the potentiating effects of melatonin observed in our experimental conditions cannot be ruled out and, therefore, further research is required to reveal other potential underlying mechanisms. To sum up, we provide evidence that in vitro melatonin strengthens 5-FU-stimulated cytotoxicity and apoptosis in HT-29 cells and, therefore, the indoleamine might be potentially used as a powerful synergistic agent so as to provide a more effective way to treat CRC.

Acknowledgements This work was supported by Junta de Extremadura (GR15051). J. Espino holds a research post-doctoral fellowship from Junta de Extremadura (jointly financed by the European Regional Development Fund (ERDF); ref. PO14011). The authors

appreciate the technical and human support provided by Facility of Bioscience Applied Techniques of SAIUEX (financed by UEX, Junta de Extremadura, MICINN, FEDER, and FSE).

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

References

1. Ferlay J, Soerjomataram I, Dikshit R et al (2015) Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 136:E359–E386. doi:10.1002/ijc.29210
2. Scholefield JH, Steele RJ, British Society For Gastroenterology, Association of Coloproctology for Great Britain and Ireland (2002) Guidelines for follow up after resection of colorectal cancer. *Gut* 51:V3–V5
3. Hardeland R, Cardinali DP, Srinivasan V et al (2011) Melatonin—a pleiotropic, orchestrating regulator molecule. *Prog Neurobiol* 93:350–384. doi:10.1016/j.pneurobio.2010.12.004
4. Trivedi PP, Jena GB, Tikoo KB, Kumar V (2016) Melatonin modulated autophagy and Nrf2 signaling pathways in mice with colitis-associated colon carcinogenesis. *Mol Carcinog* 55:255–267. doi:10.1002/mc.22274
5. García-Navarro A, González-Puga C, Escames G et al (2007) Cellular mechanisms involved in the melatonin inhibition of HT-29 human colon cancer cell proliferation in culture. *J Pineal Res* 43:195–205
6. Motilva V, García-Mauriño S, Talero E, Illanes M (2011) New paradigms in chronic intestinal inflammation and colon cancer: role of melatonin. *J Pineal Res* 51:44–60. doi:10.1111/j.1600-079X.2011.00915.x
7. Kos-Kudla B, Ostrowska Z, Kozłowski A et al (2002) Circadian rhythm of melatonin in patients with colorectal carcinoma. *Neuro Endocrinol Lett* 23:239–242
8. Schernhammer ES, Laden F, Speizer FE et al (2003) Night-shift work and risk of colorectal cancer in the nurses’ health study. *J Natl Cancer Inst* 95:825–828
9. Naziroglu M, Karaoglu A, Aksoy AO (2004) Selenium and high dose vitamin E administration protects cisplatin-induced oxidative damage to renal, liver and lens tissues in rats. *Toxicology* 195:221–230
10. Sakallı Çetin E, Naziroglu M, Çiğ B et al (2017) Selenium potentiates the anticancer effect of cisplatin against oxidative stress and calcium ion signaling-induced intracellular toxicity in MCF-7 breast cancer cells: involvement of the TRPV1 channel. *J Recept Signal Transduct Res* 37:84–93. doi:10.3109/10799893.2016.1160931
11. Koşar PA, Naziroglu M, Övey İS, Çiğ B (2016) Synergic effects of doxorubicin and melatonin on apoptosis and mitochondrial oxidative stress in MCF-7 breast cancer cells: involvement of TRPV1 channels. *J Membr Biol* 249:129–140. doi:10.1007/s00232-015-9855-0
12. Kim J-H, Jeong S-J, Kim B et al (2012) Melatonin synergistically enhances cisplatin-induced apoptosis via the dephosphorylation of ERK/p90 ribosomal S6 kinase/heat shock protein 27 in SK-OV-3 cells. *J Pineal Res* 52:244–252. doi:10.1111/j.1600-079X.2011.00935.x
13. Pariente R, Pariente JA, Rodríguez AB, Espino J (2016) Melatonin sensitizes human cervical cancer HeLa cells to cisplatin-induced cytotoxicity and apoptosis: effects on oxidative stress and DNA fragmentation. *J Pineal Res* 60:55–64. doi:10.1111/jpi.12288

14. Fan L-L, Sun G-P, Wei W et al (2010) Melatonin and doxorubicin synergistically induce cell apoptosis in human hepatoma cell lines. *World J Gastroenterol* 16:1473–1481
15. Plaimee P, Weerapreeyakul N, Barusru S, Johns NP (2015) Melatonin potentiates cisplatin-induced apoptosis and cell cycle arrest in human lung adenocarcinoma cells. *Cell Prolif* 48:67–77. doi:[10.1111/cpr.12158](https://doi.org/10.1111/cpr.12158)
16. Casado-Zapico S, Rodríguez-Blanco J, García-Santos G et al (2010) Synergistic antitumor effect of melatonin with several chemotherapeutic drugs on human Ewing sarcoma cancer cells: potentiation of the extrinsic apoptotic pathway. *J Pineal Res* 48:72–80. doi:[10.1111/j.1600-079X.2009.00727.x](https://doi.org/10.1111/j.1600-079X.2009.00727.x)
17. Uguz AC, Cig B, Espino J et al (2012) Melatonin potentiates chemotherapy-induced cytotoxicity and apoptosis in rat pancreatic tumor cells. *J Pineal Res* 53:91–98. doi:[10.1111/j.1600-079X.2012.00974.x](https://doi.org/10.1111/j.1600-079X.2012.00974.x)
18. Bejarano I, Espino J, Barriga C et al (2011) Pro-oxidant effect of melatonin in tumour leucocytes: relation with its cytotoxic and pro-apoptotic effects. *Basic Clin Pharmacol Toxicol* 108:14–20. doi:[10.1111/j.1742-7843.2010.00619.x](https://doi.org/10.1111/j.1742-7843.2010.00619.x)
19. Espino J, Bejarano I, Paredes SD et al (2011) Protective effect of melatonin against human leukocyte apoptosis induced by intracellular calcium overload: relation with its antioxidant actions. *J Pineal Res* 51:195–206. doi:[10.1111/j.1600-079X.2011.00876.x](https://doi.org/10.1111/j.1600-079X.2011.00876.x)
20. Espino J, Rodríguez AB, Pariante JA (2013) The inhibition of TNF- α -induced leukocyte apoptosis by melatonin involves membrane receptor MT1/MT2 interaction. *J Pineal Res* 54:442–452. doi:[10.1111/jpi.12042](https://doi.org/10.1111/jpi.12042)
21. Espino J, González-Gómez D, Moreno D et al (2013) Tempranillo-derived grape seed extract induces apoptotic cell death and cell growth arrest in human promyelocytic leukemia HL-60 cells. *Food Funct* 4:1759–1766. doi:[10.1039/c3fo60267b](https://doi.org/10.1039/c3fo60267b)
22. Longley DB, Harkin DP, Johnston PG (2003) 5-Fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 3:330–338. doi:[10.1038/nrc1074](https://doi.org/10.1038/nrc1074)
23. Cassidy J, Saltz L, Twelves C et al (2011) Efficacy of capecitabine versus 5-fluorouracil in colorectal and gastric cancers: a meta-analysis of individual data from 6171 patients. *Ann Oncol* 22:2604–2609. doi:[10.1093/annonc/mdr031](https://doi.org/10.1093/annonc/mdr031)
24. Wang L-H, Li Y, Yang S-N et al (2014) Gambogic acid synergistically potentiates cisplatin-induced apoptosis in non-small-cell lung cancer through suppressing NF- κ B and MAPK/HO-1 signalling. *Br J Cancer* 110:341–352. doi:[10.1038/bjc.2013.752](https://doi.org/10.1038/bjc.2013.752)
25. Rodríguez-García A, Mayo JC, Hevia D et al (2013) Phenotypic changes caused by melatonin increased sensitivity of prostate cancer cells to cytokine-induced apoptosis. *J Pineal Res* 54:33–45. doi:[10.1111/j.1600-079X.2012.01017.x](https://doi.org/10.1111/j.1600-079X.2012.01017.x)
26. Bejarano I, Redondo PC, Espino J et al (2009) Melatonin induces mitochondrial-mediated apoptosis in human myeloid HL-60 cells. *J Pineal Res* 46:392–400. doi:[10.1111/j.1600-079X.2009.00675.x](https://doi.org/10.1111/j.1600-079X.2009.00675.x)
27. Rubio S, Estévez F, Cabrera J et al (2007) Inhibition of proliferation and induction of apoptosis by melatonin in human myeloid HL-60 cells. *J Pineal Res* 42:131–138. doi:[10.1111/j.1600-079X.2006.00392.x](https://doi.org/10.1111/j.1600-079X.2006.00392.x)
28. Cos S, Garcia-Bolado A, Sánchez-Barceló EJ (2001) Direct antiproliferative effects of melatonin on two metastatic cell sublines of mouse melanoma (B16BL6 and PG19). *Melanoma Res* 11:197–201
29. Majsterek I, Gloc E, Blasiak J, Reiter RJ (2005) A comparison of the action of amifostine and melatonin on DNA-damaging effects and apoptosis induced by idarubicin in normal and cancer cells. *J Pineal Res* 38:254–263. doi:[10.1111/j.1600-079X.2005.00197.x](https://doi.org/10.1111/j.1600-079X.2005.00197.x)
30. Bennukul K, Numkliang S, Leardkamolkarn V (2014) Melatonin attenuates cisplatin-induced HepG2 cell death via the regulation of mTOR and ERCC1 expressions. *World J Hepatol* 6:230–242. doi:[10.4254/wjh.v6.i4.230](https://doi.org/10.4254/wjh.v6.i4.230)
31. Büyükcavci M, Ozdemir O, Buck S et al (2011) Effect of melatonin on the cytotoxicity of chemotherapeutic drugs in human leukemia cells. *In vivo (brooklyn)* 25:405–409
32. Kilic U, Kilic E, Tuzcu Z et al (2013) Melatonin suppresses cisplatin-induced nephrotoxicity via activation of Nrf-2/HO-1 pathway. *Nutr Metab (Lond)* 10:7. doi:[10.1186/1743-7075-10-7](https://doi.org/10.1186/1743-7075-10-7)
33. Madhu P, Reddy KP, Reddy PS (2015) Melatonin reduces oxidative stress and restores mitochondrial function in the liver of rats exposed to chemotherapeutics. *J Exp Zool A Ecol Genet Physiol* 323:301–308. doi:[10.1002/jez.1917](https://doi.org/10.1002/jez.1917)
34. Demir MG, Altıntoprak N, Aydın S et al (2015) Effect of transtympanic injection of melatonin on cisplatin-induced ototoxicity. *J Int Adv Otol* 11:202–206. doi:[10.5152/iao.2015.1094](https://doi.org/10.5152/iao.2015.1094)
35. Tan D-X, Manchester LC, Terron MP et al (2007) One molecule, many derivatives: a never-ending interaction of melatonin with reactive oxygen and nitrogen species? *J Pineal Res* 42:28–42. doi:[10.1111/j.1600-079X.2006.00407.x](https://doi.org/10.1111/j.1600-079X.2006.00407.x)
36. Xin Z, Jiang S, Jiang P et al (2015) Melatonin as a treatment for gastrointestinal cancer: a review. *J Pineal Res* 58:375–387. doi:[10.1111/jpi.12227](https://doi.org/10.1111/jpi.12227)
37. Wei J-Y, Li W-M, Zhou L-L et al (2015) Melatonin induces apoptosis of colorectal cancer cells through HDAC4 nuclear import mediated by CaMKII inactivation. *J Pineal Res* 58:429–438. doi:[10.1111/jpi.12226](https://doi.org/10.1111/jpi.12226)
38. Wang J, Guo W, Chen W et al (2013) Melatonin potentiates the antiproliferative and pro-apoptotic effects of ursolic acid in colon cancer cells by modulating multiple signaling pathways. *J Pineal Res* 54:406–416. doi:[10.1111/jpi.12035](https://doi.org/10.1111/jpi.12035)
39. Ju H-Q, Li H, Tian T et al (2016) Melatonin overcomes gemcitabine resistance in pancreatic ductal adenocarcinoma by abrogating nuclear factor- κ B activation. *J Pineal Res* 60:27–38. doi:[10.1111/jpi.12285](https://doi.org/10.1111/jpi.12285)
40. Strasser A, O'Connor L, Dixit VM (2000) Apoptosis signaling. *Annu Rev Biochem* 69:217–245. doi:[10.1146/annurev.biochem.69.1.217](https://doi.org/10.1146/annurev.biochem.69.1.217)
41. Green DR, Reed JC (1998) Mitochondria and apoptosis. *Science* 281:1309–1312
42. Gao Y, Xiao X, Zhang C et al (2017) Melatonin synergizes the chemotherapeutic effect of 5-fluorouracil in colon cancer by suppressing PI3K/AKT and NF- κ B/iNOS signaling pathways. *J Pineal Res* 62:e12380. doi:[10.1111/jpi.12380](https://doi.org/10.1111/jpi.12380)