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Effects of melatonin on doxorubicin cytotoxicity in sensitive and pleiotropically resistant tumor cells

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Abstract: Melatonin has been reported to attenuate the oxidative damage caused by doxorubicin on kidney, brain, heart and bone marrow, whereas the in vivo antitumor effects of doxorubicin were not attenuated. The effects of melatonin on doxorubicin cytotoxicity have, therefore, been examined on human normal mammary epithelium HBL-100, on mammary adenocarcinoma MCF-7, on colon carcinoma LoVo, and on mouse P388 leukemia cell lines, and on tumor cell sublines pleiotropically resistant to anthracyclines. Melatonin in the concentration range 10–2000 pg/mL causes an inhibition of the growth of the human cell lines examined which is not clearly dose-dependent and less than 25% when significant. Melatonin similarly causes minor effects on doxorubicin cytotoxicity either on the parental human cell lines or on their resistant sublines. On the contrary, 200–1000 pg/mL melatonin cause a significant and dose-dependent partial sensitization to doxorubicin of resistant P388 mouse leukemia (P388/ADR), which occurs also in vivo, as indicated by a significant increase in survival time of the hosts. Doxorubicin intracellular concentrations in P388/ADR cells are increased by melatonin, suggesting that melatonin might inhibit P-glycoprotein-mediated doxorubicin efflux from the cells. These results indicate that the use of melatonin in clinical cancer treatment should not pose the risk of an attenuation of the effectiveness of doxorubicin, and encourage the further examination of the possible reduction by melatonin of the host toxicity of antitumor chemotherapy.

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Introduction

Numerous reports are available showing that melatonin acts as an effective antioxidant agent, either via the direct scavenging of free radicals and via an influence on glutathione metabolism [Reiter, 1997a,b]. Correspondingly, reduced melatonin levels have been implicated in the oxidative degenerative processes associated with advanced age [Reiter, 1998], and the administration of exogenous melatonin was shown to reduce the damage caused by a variety of oxidative toxins [Reiter et al., 1997]. Prooxidant effects are involved in the mechanism of action of the antitumor drug doxorubicin [Gewirtz, 1999], and melatonin was accordingly found to attenuate the oxidative damage caused by this anthracycline on kidney [Montilla et al., 1998], brain [Montilla et al., 1997], and heart tissues [Morishima et al., 1998; Wahab et al., 2000]. Moreover, the myelotoxicity of doxorubicin in mice bearing TLX5 lymphoma, determined as the reduction in number of colony forming cells, was significantly attenuated when the treatment with the anthracycline was associated with the administration of pharmacological doses of melatonin, and the levels of reduced glutathione in bone marrow cells were concomitantly restored to control values [Rapozzi et al., 1998]. On the other hand, in the same mice bearing TLX5 lymphoma, the increase in life span caused by doxorubicin was not reduced by the administration of melatonin, which was devoid of any effect by itself on the survival time of the treated animals [Rapozzi et al., 1998].

It thus appears of interest to examine in more detail the effects of melatonin on the antitumor effects of doxorubicin. Direct antitumor effects of

melatonin, which could interfere with those of doxorubicin in an antagonistic, additive or synergistic way, have to be also considered. In this connection, melatonin reportedly displays antitumor effects against a variety of cancer cell lines, including MCF-7 human breast cancer cells [Cos et al., 1996a,b; Blask et al., 1997; Cos and Fernandez, 2000], 7288CTC rat hepatoma [Blask et al., 1999], SK-N-SH neuroblastoma and of BG-1 ovarian carcinoma [Cos et al., 1996c; Petranka et al., 1999], and Ishikawa endometrial cell line [Kanishi et al., 2000].

The effects of melatonin have, consequently, been determined in a broad range of physiological and pharmacological concentrations, alone or in combination with a doxorubicin concentration corresponding to an IC_{50} determined on the same cell line. The inhibitory effect of the treatments on cell proliferation has been evaluated on a normal human breast epithelium HBL-100 cell line, human breast adenocarcinoma MCF-7, human colon carcinoma LoVo and P388 mouse leukemia cell line. Since the acquired resistance to anthracyclines represents a frequent and serious clinical occurrence [Robert, 1999], tumor cell lines with acquired resistance to doxorubicin have also been included into the panel of cell lines examined. Finally, the effects of the treatments have been examined in relation to the mechanism of acquired resistance. The results obtained are hereafter reported.

Materials and methods

Cell lines and cultures

Murine leukemia P388 and P388/ADR cells (obtained from the NCI Frederick Cancer Research Facility/DCT Tumor Repository, Bethesda, MD), human breast carcinoma MCF-7 and MCF-7/ADR cells (kindly provided by Dr. K. Cowan, NCI, NIH, Bethesda, MD) and human colon adenocarcinoma LoVo and LoVo/ADR cells (kindly provided by Dr. C. Gambacorti, Milan, Italy) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. Normal human breast epithelial HBL-100 cells (obtained from the American Type Culture Collection, Rockville, MD) were cultured in McCoy's medium containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin. Cell cultures were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Measurement of cell growth and viability

Cell growth and viability were measured using the MTT assay. Cells from exponentially growing cultures were harvested and dispensed within replicate 96-well culture plates in 200 μL of medium at the concentration of 10^4 cells/well for HBL-100, MCF-7, MCF-7/ADR, LoVo, LoVo/ADR and of 2×10^3 cells/well for P388 and P388/ADR cell lines. The cells were exposed to melatonin, and 1 hr later to an IC_{50} of doxorubicin, previously determined for each cell line, as indicated in Figs. 1–5. Cell viability was measured after 72 hr of culture using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [Mosmann, 1983]. Twenty μL of a MTT stock solution (5 mg/mL in phosphate buffered saline, PBS) were added to each well; following 5 hr incubation at 37°C, supernatants were removed by careful aspiration (after centrifugation of the plates in the case of P388 and P388/ADR lines) and replaced by 200 μL of dimethyl sulfoxide (DMSO). After formazan solubilization, the optical density (OD) of each well was measured using an Automated Microplate Reader EL 311s (Bio-Tek Instruments,

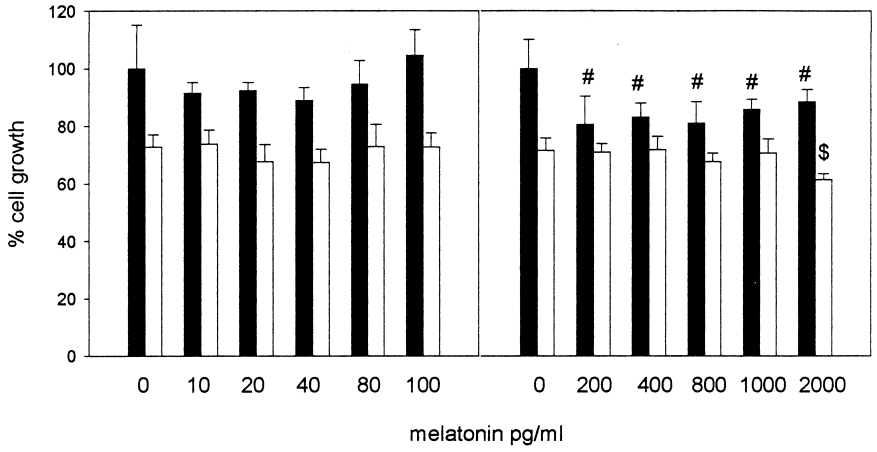

Fig. 1. Effects of melatonin and doxorubicin on HBL-100 cell growth in vitro. Cells were exposed to melatonin and 1 hr later to 10 nM doxorubicin (□) or to melatonin alone (■). Cell growth after 72 hr was measured, and is expressed as the mean (± S.D.) percent growth as compared to relevant controls. # significantly different from untreated controls, $P < 0.05$; $ significantly different from controls treated with doxorubicin, $P < 0.05$.

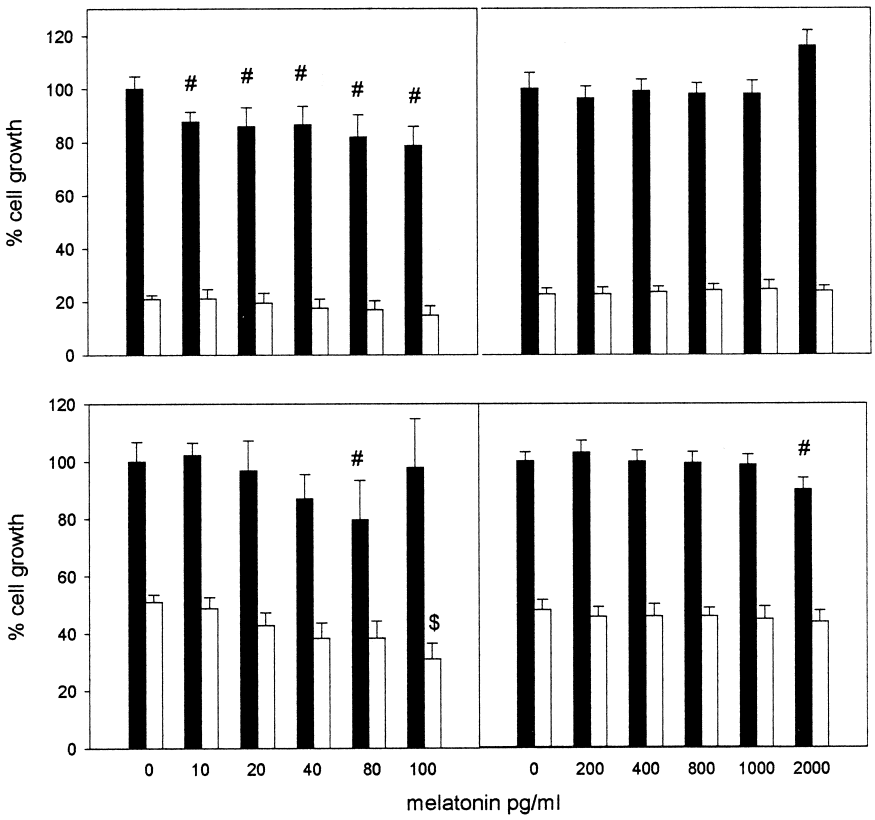

Fig. 2. Effects of melatonin and doxorubicin on MCF-7 and MCF-7/ADR cell growth in vitro. Cells were exposed to melatonin and 1 hr later to doxorubicin 1 μM for MCF-7 (upper panel), or 5 μM for MCF-7/ADR (lower panel) cell lines (□), or to melatonin alone (■). Cell growth after 72 hr was measured and is expressed as the mean (±S.D.) percent growth as compared to relevant controls. # significantly different from untreated controls, $P < 0.05$; $ significantly different from controls treated with doxorubicin, $P < 0.05$.

Inc.) with a reference wavelength of 630 nm and a test wavelength of 540 nm. The growth of viable cells is expressed as the percent ratio of OD in the treated over the untreated samples, and 8 independent measurements were averaged for each treatment.

Intracellular doxorubicin accumulation

In order to measure the effects of melatonin on doxorubicin concentration in P388 and P388/ADR cells, the natural fluorescence of doxorubicin was exploited. P388 and P388/ADR cells (10^6 cells/mL) were pre-treated with 0.1–10 ng/mL melatonin for 1 hr, 100 μM doxorubicin was then added and incubation was continued for 1.5 hr for P388, or 3 hr for P388/ADR cells. At the end of incubation period, the cells were washed twice with cold PBS, were resuspended in 2 mL of 0.3 N HCl in 50% ethanol solution, were mixed thoroughly in a vortex mixer and were centrifuged for 10 min at 700g. Doxorubicin content in the supernatant was determined fluorimetrically following the method of Bachur et al. (1970); standard curves of doxorubicin dissolved in 0.3 N HCl in 50% ethanol were used for computation of doxorubicin content.

In vivo experiments

P388 or P388/ADR lines were maintained in vivo by weekly i.p. passages of 10^6 cells into DBA/2 female mice; for experimental purposes, 10^6 viable tumor cells were injected i.p. into syngeneic BD2F1 (C57BL/6 × DBA/2 F_1) males. The animals were young mice, which were purchased from Charles River, Calco, Italy, and were maintained with a light-dark cycle of 12–12 hr (light off at 20:00 hr). The tumor cell suspension used for inoculation was prepared using cells obtained from donors similarly inoculated 7 days before; the cells were washed by centrifugation and were resuspended in PBS at a concentration of 10^6 viable cells/mL, after count with trypan blue dye. Drug treatment was performed, as indicated in Table 1, and the survival of animals was recorded.

Reagents

Melatonin was a kind gift of Professor F. Fraschini, Milan, Italy; doxorubicin was obtained from Pharmacia S.p.A., Milan, Italy, and the other reagents were purchased from Sigma-Aldrich s.r.l. Milan, Italy.

Statistical analysis

The data were subjected to the appropriate factorial ANOVA and *post hoc* Tukey test in case of significant interactions of the treatments. For survival, Kaplan Meier analysis and log rank test were performed. All analyses were performed using standard procedures implemented in the SYSTAT package.

Results

The first series of experiments was performed to determine the effects of different concentrations of melatonin, in the range from 10 to 2000 pg/mL, on cell growth and on the cytotoxicity of an IC_{50} of doxorubicin, using a panel of in vitro cultured cell lines. The lines employed included normal human breast epithelial cells HBL-100, human breast carcinoma cells MCF-7, human colon adenocarcinoma cells LoVo and murine leukemia P388; MCF-7, LoVo and P388 cell lines with acquired multidrug resistance were also examined for comparison.

On normal human breast epithelial HBL-100 cell line, melatonin in the concentration range 10–100 pg/mL is devoid of significant effects on cell growth, and has no effect on doxorubicin cytotoxicity. At the concentrations of 200–2000 pg/mL, melatonin induces a slightly significant reduction of cell growth; a marginally significant increase in doxorubicin cytotoxicity is observed with melatonin at 2000 pg/mL (Fig. 1).

On human breast carcinoma MCF-7 parental cell line, melatonin causes a marginal significant decrease of cell growth in the concentration range 10-100 pg/mL; no significant difference is observed on doxorubicin cytotoxicity by melatonin at any concentration examined. On the pleiotropically resistant MCF-7 cell line, a small significant effect is observed for melatonin at 80 and 2000 pg/mL; a significant increase in doxorubicin cytotoxicity is caused by 100 pg/mL melatonin (Fig. 2).

Limited effects are observed also on human colon adenocarcinoma LoVo cell lines. A significant inhibition is caused by 100 pg/mL melatonin on the parental line and by 1000–2000 pg/mL melatonin on the multidrug resistant line; doxorubicin cytotoxicity is slightly and significantly increased at 2000 pg/mL (Fig. 3).

Melatonin at 40–80 pg/mL moderately reduces the growth of the parental line of P388 leukemia, and does not significantly modify the effects of doxorubicin on this cell line. On the resistant P388/ADR cell line, melatonin alone significantly reduces cell growth at 400–1000 pg/mL.

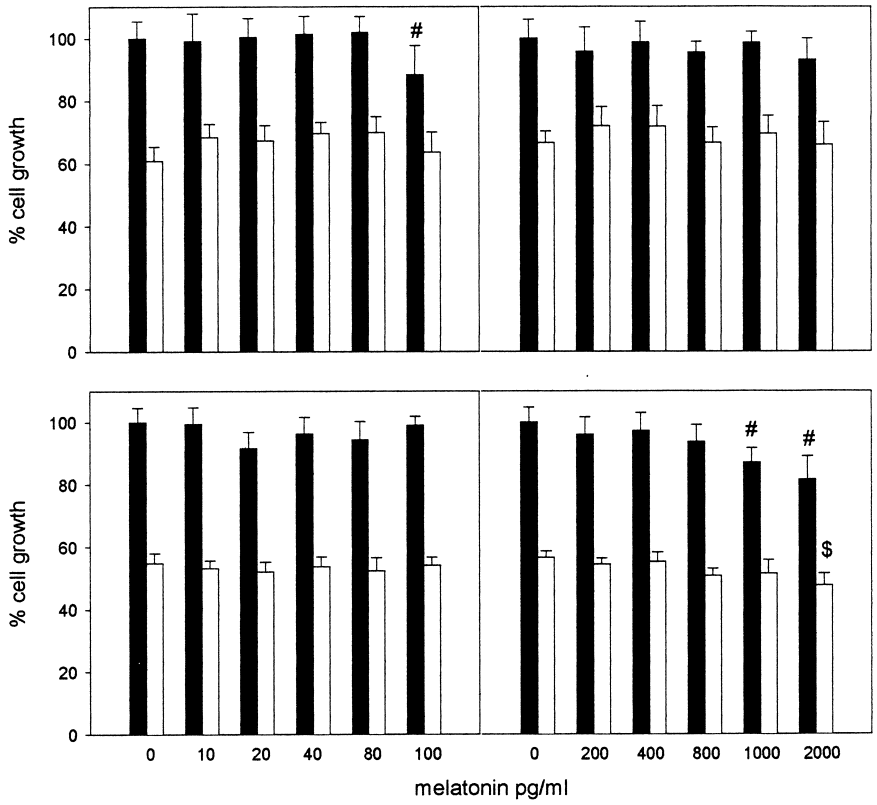

Fig. 3. Effects of melatonin and doxorubicin on LoVo and LoVo/ADR cell growth in vitro. Cells were exposed to melatonin and 1 hr later to doxorubicin 0.3 μM for LoVo (upper panel), or 10 μM for LoVo /ADR (lower panel) cell lines (□), or to melatonin alone (■). Cell growth after 72 hr was measured and is expressed as the mean (±S.D.) percent growth as compared to relevant controls. # significantly different from untreated controls, $P < 0.05$; $ significantly different from controls treated with doxorubicin, $P < 0.05$.

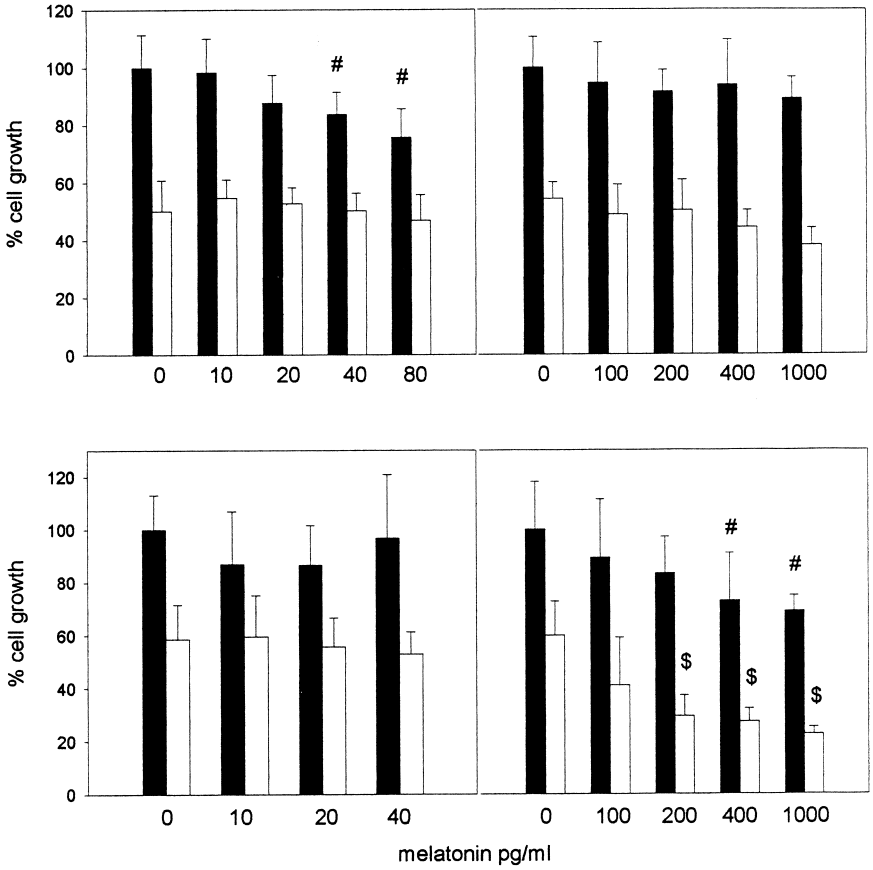

Fig. 4. Effects of melatonin and doxorubicin on P388 and P388/ADR cell growth in vitro. Cells were exposed to melatonin, and 1 hr later to doxorubicin 0.5 nM for P388 (upper panel) or 10 μM for P388/ADR (lower panel) cell lines (□), or to melatonin alone (■). Cell growth after 72 hr was measured and is expressed as the mean (±S.D.) percent growth as compared to relevant controls. # significantly different from untreated controls, $P < 0.05$; $ significantly different from controls treated with doxorubicin, $P < 0.05$.

Furthermore, a significant and more pronounced dose-dependent enhancement of doxorubicin cytotoxicity is caused by melatonin in the concentration range 200–1000 pg/mL (Fig. 4).

Data illustrated in Fig. 5 were obtained exposing P388 cells to 0.1–10 ng/mL melatonin and 100 μM doxorubicin. This concentration of the drug is higher than the IC_{50} used for the other experiments, which was determined with an exposure of the cells to the drug of 72 h, and was chosen to cause measurable intracellular drug concentrations. When P388 cells are exposed to 100 μM doxorubicin, the intracellular accumulation of the drug is not modified by 0.1–10 ng/mL melatonin. On the contrary, a significant increase in the intracellular concentration of doxorubicin is caused by 10 ng/mL melatonin on the P388/ADR resistant cell line.

The data reported in Table 1 indicate that mice bearing the resistant leukemic cell line P388/ADR do not display a significant increase in their life span when they are treated i.v. with 14 mg/kg doxorubicin. When the animals are treated with a combination of melatonin and doxorubicin, a significant increase in survival time is observed as compared with the group treated with doxorubicin only.

Discussion

Several reports show that melatonin possesses oncostatic or antiproliferative properties on a variety of tumor cell lines [Cos et al., 1996a,b,c; Blask et al., 1997, 1999; Petranka et al., 1999; Kanishi et al., 2000; Cos and Fernandez, 2000]. When studying the effects of melatonin on doxorubicin antitumor effects, the effects of melatonin alone on the specific cell line used thus have to be carefully determined. Some of the experiments of this investigation were consequently aimed at determining the antiproliferative effects of melatonin in a panel of normal and tumor cells in culture. The results obtained examining a broad range of physiological and pharmacological concentrations of melatonin were substantially negative. In fact, although a statistically significant inhibition of cell growth in vitro was observed at certain melatonin dosages on normal human breast epithelium HBL-100, human breast adenocarcinoma MCF-7, human colon carcinoma LoVo and P388 mouse leukemia cell lines, a lack of a clear dose dependence for the inhibitory effects was evident, and when significant effects were seen, they were of low statistical significance. A full review of the role of melatonin in oncology cannot be discussed here. However,

the lack of a clear antiproliferative action presently observed for melatonin on the tumor lines employed, was previously reported also for B16 melanoma [Helton et al., 1993], for HeLa cervical carcinoma, MG-63 osteosarcoma and also for MCF-7 cell lines [Panzer et al., 1998]. The differences resulting for MCF-7 cells have been already investigated, considering the established role of melatonin in mammary pathological growth [Cos and SanchezBarcelo, 2000a], and might be attributed to the estrogen receptor status of the cells, as well as to different experimental conditions, such as the pattern of exposure to melatonin and the cell proliferation rate which might account for the differences also concerning other cell lines [Cos and SanchezBarcelo, 2000b].

A second series of experiments examined the possible influences of melatonin on the cytotoxic effects of doxorubicin in vitro. The association of melatonin, at the same wide range of physiological and pharmacological concentrations used in the previous experiments, with an IC_{50} of doxorubicin, did not significantly increase the cytotoxicity of the anthracycline on any of the parental lines examined. This finding is in agreement with the results obtained in mice bearing TLX5 lymphoma, showing that the antitumor action (increase in survival time) caused by doxorubicin was not reduced by the concomitant administration of large doses of melatonin, which were devoid of any effects on the survival time of the animals by themselves. At the same time, a significant reduction of the myelotoxicity of doxorubicin was observed when the mice were treated with doxorubicin in combination with melatonin [Rapozzi et al., 1998]. A protection of bone marrow haematopoietic function by melatonin was also observed in mice bearing Lewis lung carcinoma when treated with cyclophosphamide or etoposide, while the cytotoxic effects of both drugs were not attenuated [Maestroni et al., 1994]. Moreover, the pretreatment with melatonin of Ehrlich ascites carcinoma-bearing mice prior to doxorubicin administration even improved the antitumor activity of this anthracycline, protecting at the same time the animals from the cardiotoxicity induced by doxorubicin [Wahab et al., 2000]. The precise mechanism by which melatonin does not reduce or even increase the antitumor action of doxorubicin, while it attenuates toxicity for normal tissues of the host in vivo, remains to be investigated.

It thus appears that melatonin does not attenuate the antitumor action of the prooxidant agents doxorubicin and etoposide, and of the alkylating nitrogen mustard cyclophosphamide, under several in vitro and in vivo conditions. These results suggest that the use of melatonin in cancer patients treated with chemotherapy should be safe, in the sense that it should not reduce the efficacy of chemotherapy. Indeed, in a large group of advanced cancer patients treated with polychemotherapy including the prooxidant agents doxorubicin, etoposide, mitoxantrone, the concomitant administration of melatonin significantly increased the 1-year survival rate and the objective tumor responses, and significantly reduced the toxicity of the treatments [Lissoni et al., 1999c]. However, a protection of the host from the toxicity of chemotherapy, which might be expected considering the experimental data mentioned above, was not observed in a group of previously untreated patients with inoperable lung cancer receiving melatonin in addition to chemotherapy with carboplatin and etoposide [Ghielmini et al., 1999].

Finally, the effects of melatonin alone or in association with doxorubicin have been determined in cultures of pleiotropically resistant MCF-7, LoVo and P388 sublines. On MCF-7 and

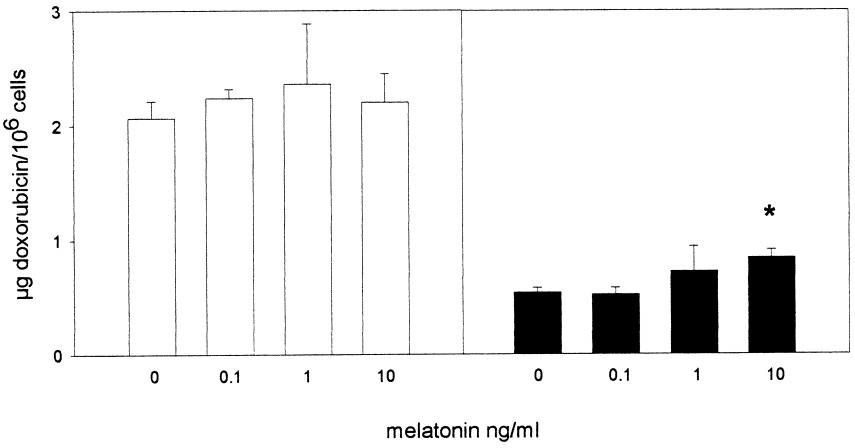

Fig. 5. Effects of melatonin on intracellular accumulation of doxorubicin in P388 and in P388/ADR cells. The cells were preincubated with the indicated concentration of melatonin, were further incubated for 1.5 hr (P388) (□), or 3 hr (P388/ADR) (■) with 100 μM doxorubicin, and doxorubicin intracellular concentration was measured fluorimetrically. Each value is the mean (±S.D.) of 3 separate determinations. * significantly different from untreated controls, $P < 0.05$.

Table 1. Effects of melatonin on the survival time of mice implanted with P388/ADR and treated with doxorubicin

Treatment				Log rank	
ADR	MELA	Group size	Mean survival time (days)		
−	−	7	12.9[a]	*a* vs. *b*	$P = 0.04$
+	−	7	11.3[b]	*b* vs. *c*	$P = 0.03$
+	+	6	13.9[c]		

Groups of 8 mice were implanted with 10^6 P388/ADR cells, and after 24 hr were treated with melatonin (MELA) 10 mg/kg s.c. at light off (20:00 hr) and/or with doxorubicin (ADR) 14 mg/kg i.v. 1 hr later, as indicated; melatonin treated animals also received melatonin in their drinking water (100 μg/mL) until death. The day of death was recorded and survival was analyzed with Kaplan Meier and log rank methods.

LoVo cells, a significant but marginal inhibition was observed at single concentrations of melatonin; the cytotoxicity of doxorubicin on these resistant cell lines was also slightly increased by melatonin at some dosages. On the other hand, a slightly more pronounced dose-dependent inhibition was caused on P388/ADR mouse leukemia by 400–1000 pg/mL melatonin. Moreover, a dose-dependent sensitization of this multi-drug resistant line towards doxorubicin was significantly induced by 200–1000 pg/mL melatonin. This partial reversal of resistance in P388/ADR cells was also observed in vivo, as a dose of doxorubicin which was ineffective by itself, caused a significant increase in life span when combined with the administration of melatonin. The mechanism of the partial reversal of multi-drug resistance by melatonin might consist in a greater uptake or in a reduced efflux of doxorubicin in P388/ADR cells, since the intracellular content of the anthracycline was increased after preincubation with melatonin. A reduction in doxorubicin efflux appears more likely, as it might be attributed to an inhibition by melatonin of P-glycoprotein which has been shown to be responsible for pleiotropic resistance in many cell lines, including P388/ADR [Inaba and Johnson, 1978].

In conclusion, the data reported so far indicate for the panel of normal and tumor cells presently examined that melatonin, even at high supra-physiological concentrations, possess a marginal oncostatic action and does not reduce the cytotoxicity of doxorubicin. Similar substantially negative results were obtained with pleiotropically resistant sublines, whith the exception of P388 mouse leukemia cell line, whose multi-drug resistant subline was partially sensitized by melatonin to the in vitro and in vivo cytotoxic action of doxorubicin. Altogether, the data presented appear to indicate that the clinical use of melatonin in cancer treatment should not pose the risk of an attenuation of the effectiveness of other concurrent therapies, such as chemotherapy. These results and considerations thus do not appear to discourage the further examination of the possible benefits deriving from melatonin treatment, such as the attenuation of the systemic toxicity of antitumor chemotherapy [Lissoni et al., 1999c], or the immunomodulatory properties [Liebmann et al., 1997] which might account for the beneficial clinical effects repeatedly reported by Lissoni and coworkers [Barni et al., 1995; Lissoni et al., 1996a,b, 1999a,b; Lissoni, 1997].

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