Synergistic Cytotoxicity of Melatonin and New-generation Anticancer Drugs Against Leukemia Lymphocytes But Not Normal Lymphocytes

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Abstract. *The present study demonstrates specific sensitization of leukemia lymphocytes towards anticancer drugs using melatonin and clarifies the role of reactive oxygen species (ROS) for induction of apoptosis. The study covers four conventional and 11 new-generation anticancer drugs. Four parameters were analyzed simultaneously in leukemia and normal lymphocytes treated with drug, melatonin, or their combination: cell viability, induction of apoptosis, level of reactive oxygen species (ROS), and level of protein–carbonyl products. Almost all investigated combinations of melatonin with new-generation anticancer drugs were characterized by synergistic cytotoxicity towards leukemia lymphocytes, while the combinations with conventional drugs exhibited additive or antagonistic effects on cell viability. In leukemia lymphocytes, the additive cytotoxicity of doxorubicin plus melatonin was accompanied by low levels of ROS and protein–carbonyl products, as well as by suppression of apoptosis. In normal lymphocytes, none of the studied parameters changed significantly compared to cells treated with doxorubicin only. The combinations of everolimus plus melatonin and barasertib plus melatonin exhibited impressive synergistic cytotoxic effects on leukemia lymphocytes but did not affect the viability of normal lymphocytes. In leukemia cells, the synergistic cytotoxicity was accompanied by strong induction of apoptosis but a decrease of ROS to a level below that of the control. In normal lymphocytes, these combinations did not affect the level*

of ROS nor of protein–carbonyl products, and did not induce apoptosis. The data suggest that melatonin is a promising supplementary component in chemotherapy which allows the therapeutic doses of anticancer drugs to be reduced, minimizing their side-effects.

It is widely accepted that the efficiency of conventional anticancer drugs is due to induction of oxidative stress in cancer cells. However, their harmful side-effects are also attributed to the increased production of reactive oxygen species (ROS) and disruption of the redox-homeostasis of normal cells and tissues (1-4). In the past two decades, a large number of studies have shown that natural or synthetic compounds which modulate the cellular redox status exhibit anticancer effects. The data are summarized in several excellent review articles published recently (5-8). Some of the described redox modulators induce production of ROS, but in most cases, their anticancer activity is related to the influence on signaling pathways for regulation of physiological processes, rather than by induction of strong oxidative stress and damage to biomacromolecules (5-10).

The pituitary hormone melatonin (*N*-acetyl-5-methoxytryptamine) is of great interest as an endogenous redox modulator with anticancer activity. Melatonin is responsible for the synchronization of the circadian rhythm of physiological functions in mammals (11-13). It has been shown that melatonin plays a direct role in mitochondrial homeostasis (11, 14-16), which may explain its protective effect on variety of diseases accompanied by mitochondrial dysfunction as a primary or secondary cause of the pathology, such as: Parkinson's disease, Alzheimer's disease, epilepsy, aging, ischemia-reperfusion and sepsis (11, 14, 17-20). Melatonin also acts as an antioxidant – directly as a scavenger of ROS (14, 15, 17, 18, 21), and indirectly through regulating the expression and activities of antioxidant enzymes and nitric oxide synthase (NOS) (11, 22-24).

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In pharmaceutically-relevant doses, melatonin inhibits tumor growth and has a potential therapeutic value in treating breast cancer, prostate cancer, melanoma and gastrointestinal cancer (11, 14, 25-27). For example, a strong pro-apoptotic effect of melatonin (at 100 nM) was observed in MCF-7 breast tumor cells after 20 hours of treatment (28). The effect was accompanied by disruption of mitochondrial respiration and ~40-60% loss of MCF-7 cells. Melatonin also inhibited growth and induced apoptosis of MOLT-3 human leukemia cells (29). In this case, the intrinsic apoptotic pathway has a key role in melatonin-induced cell death because of activation of the caspase cascade, an increase of the BAX/BCL2 ratio and of the cytochrome *c* level in the cytosol (29). Up-regulation of BAX in response to melatonin has been reported in HL-60 human myeloid cells, pancreatic carcinoma, and HepG2 liver carcinoma cells (30-32). Both the intrinsic (mitochondrial-mediated) and the extrinsic (receptor-mediated) pathways of apoptosis are activated by melatonin in cancer but not in normal cells (27, 30, 33, 34). It seems that melatonin has differential effects on the normal cells especially related to the regulation of apoptosis. The reason for pro-apoptotic selectivity and efficiency of melatonin in cancer is still unclear. Most likely, melatonin affects biochemical pathways that are unique to cancer. These findings suggest the potential of melatonin in the preferential killing of cancer cells and preservation of normal cells and tissues – a dream therapeutic strategy for decades.

The aim of the present study was to clarify the role of melatonin as a redox modulator and chemosensitizer in cancer and normal cells. We investigated: (i) the possibility of sensitizing leukemia lymphocytes to anticancer drugs using melatonin in pharmacologically permissible doses; (ii) combinations of drugs and melatonin with synergistic cytotoxic effect on leukemia lymphocytes and determined their cytotoxicity towards normal lymphocytes; (iii) and the role of ROS in the induction of apoptosis and cytotoxicity by those combinations. The study covers 15 anticancer drugs – four conventional and 11 new-generation.

Materials and Methods

Cells and treatment protocol. The experiments were performed on Jurkat leukemia lymphocytes, derived from patients with acute lymphoblastic leukemia, as well as on normal lymphocytes, isolated from healthy blood donors using Lymphosepar-I (Immuno-Biological Laboratories Co., Fujioka, Japan). The cells were cultured in RPMI-1640 medium, supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin), in a humidified atmosphere at 37°C with 5% CO₂. All cells were collected by centrifugation (1,000 × *g*, 10 min) and replaced in a fresh medium without antibiotics before treatment with anticancer drugs.

The drugs were dissolved in dimethyl sulfoxide (DMSO; suitable for cell cultures) or phosphate-buffered saline (PBS; 10 mM, pH 7.4).

The final concentration of DMSO in the cell suspension did not exceed 1%. At this concentration, DMSO did not influence cell viability.

The cells (1×10⁶ cells/ml) were incubated with melatonin, drug, or drug plus melatonin at the following concentrations: 250 µM melatonin (Sigma-Aldrich, Steinheim, Germany), 0.25 µM palbociclib (Selleckchem, Houston, TX, USA), 0.5 µM PI-103, 5 µM (Selleckchem), everolimus (Selleckchem), 0.5 µM lonafarnib (Selleckchem), 0.1 µM ABT-737 (Selleckchem), 0.1 µM doxorubicin (Sigma-Aldrich), 0.5 µM bleomycin (Nippon Kayaku Co., Tokyo, Japan), 0.1 µM AZD-7762 (Sigma-Aldrich), 0.01 µM MLN-2238 (Selleckchem), 0.025 µM MG-132 (Wako, Tokyo, Japan), 10 µM lomustine (Sigma-Aldrich), 2.5 µM cisplatin (Selleckchem), 0.025 µM BEZ-235 (Selleckchem), 0.01 µM bortezomib (Selleckchem), 0.05 µM or 0.01 µM barasertib (Selleckchem). Drugs were applied to the cells at these concentrations (single dose) and incubated for different time intervals in a cell incubator. At each time interval, aliquots were used for cell viability assay. The selected concentrations of drugs and melatonin (applied separately) induced about 20% inhibition of cell growth.

Cell viability assay. Cell viability was analyzed using trypan blue staining and Countess™ Automated Cell Counter (Invitrogen, OR, USA) at very precise standardization of the measurements. Three independent experiments (with two repetitive measurements) for each experiment were performed for each sample. Non-treated cells were used as controls. The data are presented as the mean±SD.

Intracellular ROS assay. The amount of ROS was analyzed using OxiSelect™ *In vitro* ROS/RNS Assay Kit – Green Fluorescence (Cell Biolabs, Inc., San Diego, CA, USA). The method is based on the use of fluorogenic probe 2',7'-dichlorodihydrofluorescein DiOxyQ (DCFH-DiOxyQ). In the cytosol, the probe is deacetylated to the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH). DCFH reacts with ROS and reactive nitrogen species (RNS) (predominantly H₂O₂, ROO•, NO, ONOO⁻) with formation of fluorescent product 2',7'-dichlorodihydrofluorescein (DCF). The intensity of DCF fluorescence (λ_{ex}=480 nm, λ_{em}=530 nm) is proportional of the amount of ROS/RNS in the biological sample.

The amount of ROS/RNS was calculated by calibration curve based on DCF standard solution in PBS. The measurements were performed on a Tecan Infinite F200 PRO (Tecan Austria GmbH, Mannedorf, Austria) microplate reader.

Briefly, the cells (1×10⁶ cells/ml) were collected by centrifugation (1,000 × *g* for 10 min) and lysed by using 300 µl of 0.1% sodium dodecylsulfate (SDS; dissolved in PBS) within 30 min on ice. The lysates were adjusted to equal protein concentration (in the range 1-10 mg/ml) using PBS. Protein concentration was analyzed by Bradford assay. Each sample was subjected to ROS/RNS assay, according to the manufacturer's instruction.

Protein-carbonyl assay. The amount of protein-carbonyl products were analyzed using OxiSelect™ Protein Carbonyl Spectrophotometric Assay Kit (Cell Biolabs, Inc.). The most common products of protein oxidation in biological samples are the protein-carbonyl derivatives of proline, arginine, lysine and threonine, which are chemically stable and serve as markers of oxidative stress. The analysis of these products is based on derivatization of the carbonyl groups with dinitrophenylhydrazine with formation of protein-hydrazone. The amount of protein-hydrazone was detected spectrophotometrically at 375 nm.

Briefly, the cells (1×10^6 cells/ml) were collected by centrifugation ($1,000 \times g$, 10 min) and lysed using 300 μ l of 0.1% SDS (dissolved in PBS) within 30 min on ice. The lysates were adjusted to equal protein concentration (in the range 1-10 mg/ml) using PBS. Protein concentration was analyzed by Bradford assay. Each sample was subjected to protein-carbonyl assay, according to the manufacturer's instruction. Oxidized bovine serum albumin was used as a standard.

Apoptosis assay. The induction of apoptosis was analyzed by the expression of phosphatidylserine (PSer) on the cell surface, using FITC-Annexin V Apoptosis Detection Kit (BioVision, Milpitas, CA, USA).

Briefly, the cells (1×10^6 cells/ml) were incubated with drug, melatonin or their combination, under the conditions mentioned above. At different time-points, the cells were collected by centrifugation ($1,000 \times g$, 10 min), washed twice with PBS containing 2.5 mM CaCl_2 (annexin V-binding buffer) and re-suspended in the same buffer. One hundred microliters of the suspension were incubated with 5 μ l of fluorescein isothiocyanate (FITC)-annexin V for 10 min at room temperature in the dark. The cells were washed three times with annexin V-binding buffer and were finally re-suspended in 500 μ l of the same buffer. FITC-annexin V bound to PSer exposed on the cell surface was detected spectrofluorimetrically at $\lambda_{\text{ex}}=488$ nm and $\lambda_{\text{em}}=535$ nm, using a Tecan Infinite F200 PRO (Tecan Austria GmbH) microplate reader.

Statistical analysis. The results are expressed as the mean $\pm$ SD. Statistical analysis was performed with Student's *t*-test. A value of $p < 0.05$ was considered significant.

Results and Discussion

Effect of melatonin on viability of leukemia and normal lymphocytes. The purpose of the first stage of our study was to select an optimal concentration of melatonin for application in combination with anticancer drugs. The concentration of melatonin, which led to about 20-30% cytotoxicity against leukemia lymphocytes after 48-h incubation was assumed to be 'optimal', since this cytotoxic effect allows an assessment of synergism, additivity and antagonism after combining melatonin with anticancer drug.

The data in Figure 1 demonstrate the viability of leukemia and normal lymphocytes after treatment with melatonin at different concentrations for different time intervals (24-72 hours). Melatonin in concentrations up to 500 μ M had no effect on the viability of normal lymphocytes (Figure 1B). However, in concentrations over 100 μ M, it possessed low but significant cytotoxicity towards leukemia lymphocytes (Figure 1A). These observations confirm the results published in the literature about the cytotoxicity of melatonin towards leukemia cells and its protective effect on human lymphocytes (29, 30, 35-38).

Based on the data in Figure 1, we chose to continue our experiments with melatonin at a concentration of 250 μ M. At this concentration, melatonin causes about 20% inhibition of cell proliferation (IC_{20}) of Jurkat cells (Figure 1A) and

Figure 1. Effect of melatonin on viability of Jurkat leukemia lymphocytes (A) and normal lymphocytes (B) after different incubation times, showing a concentration-dependent effect. The data are the mean $\pm$ SD from six independent experiments. The red arrow indicates the concentration of melatonin (250 μ M) selected for further experiments in combination with anticancer drugs.

did not exhibit cytotoxic effect on normal lymphocytes after 48-hour incubation (Figure 1B).

Effect of melatonin on cytotoxicity of anticancer drugs towards leukemia lymphocytes. In the next stage of the study, Jurkat cells were treated with melatonin and anticancer drugs, separately and in combination, for different time intervals (24, 48, and 72 h). The purpose of this experiment was to find combinations with synergistic cytotoxic effect on leukemia cells. The data are shown in Figure 2. To distinguish the synergistic effect from antagonistic/additive effects, we calculated the effect of each combination on cell proliferation as a percentage of the effect of the respective drug applied alone and compared it with the effect of that when melatonin was applied alone, as described previously (9). The red line in Figure 2B indicates the effect of melatonin alone on cell proliferation. In the case of drug plus melatonin, the data located to the left of the red line reflect synergistic cytotoxic effects, while the data located to the right of the red line represent antagonistic effects. All data matching the red line reflect an additive effect. The cytotoxicity of each drug (applied separately at the selected concentration) ranged from

Figure 2. A: Effect of melatonin and anticancer drugs on cell proliferation of Jurkat cells after incubation for 24, 48 and 72 h. Incubation conditions: 1×10^6 cells/ml, melatonin (250 μ M) with/without drug (at concentration described in the Materials and Methods), at 37°C in humidified atmosphere. The data are the mean $\pm$ SD from six independent experiments. B: Effect of combination of drug plus melatonin on cell proliferation as a percentage of the effect of drug applied alone. The red line indicates the effect of melatonin on cell proliferation of cancer cells as a percentage of the control (untreated cells). *In this experiment, the concentration of barasertib was 50 nM.

about 10-20% after 24-h incubation to about 20-30% after 48-h and 72-h incubation.

Half of the combinations (drug plus melatonin) were characterized by synergistic cytotoxic effects on Jurkat cells compared to cells treated with drug only (Figure 2B). The synergism increased with incubation time. The best synergistic

cytotoxic effects were observed on Jurkat cells treated with melatonin in combination with: bortezomib, barasertib, MG-132, MLN-2238, everolimus, lonafarnib, and palbociclib.

Two of these synergistic combinations were selected for the next stage of the study: everolimus plus melatonin, and barasertib plus melatonin. In our recent study, we observed

Figure 3. Effects of melatonin (250 μ M), doxorubicin (0.1 μ M) and their combination on cell viability, induction of apoptosis, level of reactive oxygen species (ROS) and level of protein-carbonyl products in Jurkat leukemia cells (A) and normal lymphocytes (B), after 24- and 48-h incubation at 37°C in humidified atmosphere. The data are the mean $\pm$ SD from three independent experiments. * p <0.05, ** p <0.01, # p <0.001 versus corresponding control.

that everolimus and barasertib applied alone did not induce production of ROS in leukemia and normal lymphocytes (10). In addition, we selected a combination of melatonin with a conventional anticancer drug, doxorubicin, which is known to increase ROS in cancer and normal cells (1, 10, 39).

Effect of melatonin on doxorubicin-induced cytotoxicity, apoptosis, production of ROS and protein-carbonyl products in leukemia and normal lymphocytes. The purpose of this stage of the study was to investigate the effect of this combination on viability of normal lymphocytes and to clarify whether it was accompanied by induction of apoptosis through ROS-dependent or ROS-independent mechanism(s) in both cell types (leukemia and normal).

Doxorubicin at 0.1 μ M was equally cytotoxic towards leukemia and normal lymphocytes (Figure 3). Cytotoxicity increased with the time of incubation, from ~5-10% after 24-h to ~25% after 48-h. This was accompanied by an increase in ROS and protein-carbonyl products in both cell types, as well as by induction of apoptosis, especially after 48-h incubation. The effects of doxorubicin were more apparent in leukemia lymphocytes than in normal lymphocytes.

Melatonin, at 250 μ M, was slightly cytotoxic towards Jurkat cells (~10% after 48-h incubation), but not towards

normal lymphocytes. In leukemia cells, cytotoxicity was accompanied by increased production of ROS and induction of apoptosis, but without effect on the level of protein-carbonyl products. This observation is a little unexpected in the context of the commonly accepted belief that melatonin is an antioxidant. Recently, some studies reported that melatonin increased production of ROS and induced apoptosis of leukemia cells (HL-60, CMK, Jurkat, MOLT-4) (40, 41). Radogna *et al.* also described a pro-oxidant effect of melatonin in leukocytes which was mediated by lipoxygenase *via* stimulation of arachidonic acid metabolism (42). A number of studies on other cancer cell lines also reported pro-oxidant effects of melatonin at physiologically relevant doses (43-47). Obviously, in some cases, melatonin acts as an antioxidant but in other cases as a pro-oxidant. Sanchez-Sanchez *et al.* proposed a very interesting hypothesis that the inhibition of proliferation by melatonin correlates with a decrease on intracellular ROS and increase of antioxidant defence systems, while induction of cell death correlates with an increase of intracellular ROS and decrease of antioxidant defences (45). In this context, melatonin should be considered as a redox modulator, not as a classical antioxidant.

Moreover, when referring to ROS-mediated anticancer effects, it is necessary to consider and specify the type of

Figure 4. Effects of melatonin (250 μ M), everolimus (5 μ M) and their combination on cell viability, induction of apoptosis, level of reactive oxygen species (ROS) and level of protein-carbonyl products in Jurkat leukemia cells (A) and normal lymphocytes (B), after 24- and 48-h incubation at 37°C in humidified atmosphere. The data are the mean $\pm$ SD from three independent experiments. * p <0.05, ** p <0.01, # p <0.001 versus corresponding control.

Figure 5. Effects of melatonin (250 μ M), barasertib (0.01 μ M) and their combination on cell viability, induction of apoptosis, level of reactive oxygen species (ROS) and level of protein-carbonyl products in Jurkat leukemia cells (A) and normal lymphocytes (B), after 24- and 48-h incubation at 37°C in humidified atmosphere. The data are the mean $\pm$ SD from three independent experiments. * p <0.05, ** p <0.01, # p <0.001 versus corresponding control.

ROS. The ratio between main intracellular ROS (*e.g.* superoxide and H_2O_2) might be crucial for the choice at cell signaling, proliferation or apoptosis, as well as for the metastatic potential of cancer cells. Dual effects of superoxide and H_2O_2 on cell-signaling regulation have been established and discussed in the literature. An interesting hypothesis has described by Pervaiz and Clement (48). The authors consider superoxide as 'oncogenic' ROS and H_2O_2 as 'oncosuppressive' ROS. They suggested that when the ratio is predominantly in favor of superoxide the cellular state inhibits apoptosis and promotes cell survival. If the ratio changes in favor of H_2O_2 , this creates an intracellular environment suitable for induction of apoptosis and cell death. This hypothesis is well-grounded by numerous experimental and empirical observations on cells, animals and human, which indicate that: (i) almost all types of cancer cells are characterized by overproduction of superoxide and permanent oxidative stress due to their mitochondrial dysfunction and up-regulation of NADPH-dependent oxidase complex (NOX); (ii) H_2O_2 induces apoptosis in abnormal concentrations; and (iii) the overexpression of superoxide dismutase (SOD) and SOD mimetics abrogate the growth and proliferation of cancer cells *in vitro* and *in vivo*, which is indirect evidence for the oncosuppressive role of H_2O_2 (2, 48-51). It seems that superoxide and H_2O_2 activate different signaling pathways with opposite consequences for cells – survival or apoptosis.

The data in Figure 3 demonstrate that the combination of doxorubicin plus melatonin exhibited additive cytotoxic effect on both cell types – leukemia and normal. In Jurkat cells, the cytotoxicity was accompanied by low (control) levels of ROS and protein-carbonyl products, as well as by decreased expression of P^{Ser} on the cell surface, which indicates a suppression of apoptosis (Figure 3A). In normal lymphocytes, none of the parameters changed significantly compared to cells treated with doxorubicin only (Figure 3B).

Song *et al.* also reported that the increase in intracellular level of ROS in A549 lung cancer cells, which is necessary for the cytotoxicity of doxorubicin, was completely abolished upon melatonin co-treatment (46, 47). Granzotto *et al.* investigated the effect of melatonin on doxorubicin-induced cytotoxicity towards human normal mammary epithelium HBL-100, mammary adenocarcinoma MCF-7, colon carcinoma LoVo, and mouse P388 leukemia cell lines, as well as on cancer cell sub-lines which are resistant to anthracyclines (53). Their data demonstrate that melatonin had minor effects on doxorubicin-induced cytotoxicity in parental human cell lines and their resistant sub-lines. In contrast, melatonin led to significant and dose-dependent sensitization to doxorubicin of resistant P388 leukemia cells (P388/ADR), which also occurs *in vivo*, as indicated by a significant increase in survival time of the hosts. These effects were accompanied by inhibition of P-glycoprotein-

mediated doxorubicin efflux from cells co-treated with melatonin. In addition, melatonin abolished the disturbance of mitochondrial potential that occurs in doxorubicin-treated cells (34, 54).

At the same time, there is evidence which has shown that melatonin can be concomitantly administered with drugs to improve the therapeutic effect or to reduce adverse side-effects (54-56). For example, Fic *et al.* reported that melatonin increased the cytotoxicity of doxorubicin towards human keratinocytes (primary culture), A569 non-small cell lung cancer, and HEP2 laryngeal cancer cell lines, significantly reducing cell numbers and inducing apoptosis in concentration-dependent manner (56). Plaimee *et al.* also showed that melatonin significantly augmented the cytotoxicity of cisplatin towards SK-LU-1 human lung adenocarcinoma cell line and increased the population of apoptotic cells by increasing mitochondrial membrane depolarization, activating the caspases cascade and inducing cell-cycle arrest in the S-phase, compared to cells treated with cisplatin alone (57).

Effect of melatonin on everolimus-induced cytotoxicity, apoptosis, production of ROS and protein-carbonyl products in leukemia and normal lymphocytes. We obtained more impressive data after combining melatonin with new-generation chemotherapeutics, such as everolimus and barasertib. Everolimus is an inhibitor of the mammalian target of rapamycin (mTOR) pathway which plays a fundamental role in the regulation of cell viability, translational initiation, and cell-cycle progression. This drug is usually used in the clinic as an immunosuppressant to prevent rejection of organ transplants. In the past 10 years, it was found that everolimus also possesses anticancer activity (58-64). It sensitizes cancer cells to other anticancer drugs, as well as preventing the development of multidrug resistance through altering the balance between pro-apoptotic and anti-apoptotic factors (61, 64). Certain researchers have shown that the anticancer effect of everolimus was not mediated by increasing production of ROS (65), but others suggest the involvement of ROS in this process (66).

Our study shows that everolimus, at 5 μ M, exhibited a slight cytotoxic effect (~10-15% after 48-hour incubation) towards leukemia lymphocytes but not towards normal lymphocytes (Figure 4). In Jurkat cells, the cytotoxicity of everolimus applied alone was accompanied by strong induction of apoptosis, but relatively slight (insignificant) increase of the level of ROS, and without change in the level of protein-carbonyl products. Melatonin increased the cytotoxicity of everolimus by up to ~60-70% after 48-h incubation with leukemia lymphocytes but the combination did not affect the viability of normal lymphocytes. In Jurkat cells, the combination exhibited a very strong induction of apoptosis (~8-fold that of the control) but this effect was

accompanied by a reduction of ROS even below that of the control (Figure 4A). In normal lymphocytes, the combination of everolimus plus melatonin did not affect the level of ROS nor the level of protein–carbonyl products, and did not induce apoptosis (Figure 4B).

Our data indicate that the high levels of ROS are not obligatory for induction of apoptosis in cancer cells, and particularly in leukemia lymphocytes. Obviously, the impressive induction of apoptosis and synergistic cytotoxicity of the combination of everolimus plus melatonin towards Jurkat cells is a result of ROS-independent mechanism(s).

Recently it was reported that melatonin down-regulates expression of *mTOR*, as well as other oncogenes [*e.g.* epidermal growth factor receptor 2 (HER2), p38 mitogen-activated protein kinase (MAPK), protein kinase B (p-AKT) and early growth response protein 3 (EGR3)], and up-regulates tumour-suppressor genes [*e.g.* glypican 3 (GPC3)] (67, 68). This could explain the synergistic cytotoxicity of the combination of everolimus plus melatonin towards leukemia lymphocytes, as well as the differential effects on cell viability of leukemia and normal lymphocytes.

Effect of melatonin on barasertib-induced cytotoxicity, apoptosis, production of ROS and protein–carbonyl products in leukemia and normal lymphocytes. Barasertib belongs to a new class of pyrazoloquinazolines, selective inhibitors of aurora B kinase, discovered and described in 2007 (69). Currently, the aurora kinases are the subject of considerable interest as targets for the development of new anticancer agents. Little is known on the molecular mechanisms of action of barasertib, except that it inhibits aurora B kinase, provokes cell-cycle arrest and apoptosis, as well as enhancing the response to chemotherapy (70-72).

In our study, both cell types were treated with a very low concentration of barasertib (10 nM). At this concentration, barasertib when applied alone did not affect the viability of either cell type (Figure 5). However, the combination of barasertib plus melatonin induced a strong cytotoxic effect on Jurkat cells (~45-50%), which was accompanied by a strong induction of apoptosis (6-fold that of the control) and a relatively moderate enhancement of ROS and protein–carbonyl levels, as detected after 48-hour incubation (Figure 5A). In normal lymphocytes, the combination did not induce apoptosis and nor affected the level of protein–carbonyl products, but did induce a slight production of ROS (Figure 5B).

Clearly, the extremely strong induction of apoptosis and differential synergistic cytotoxicity of barasertib plus melatonin do not require production of ROS. Other mechanisms underlie these effects.

It has been demonstrated that aurora B kinase, which exists in a complex with survivin and mTOR, synergistically regulates survival and proliferation of leukemia and lymphoma cells *via* cross-talk with AKT, mTOR and

NOTCH signaling pathways (73, 74). Moreover, several studies have reported that aurora kinase inhibitors induce cell-cycle arrest and apoptosis of cancer cells through p38 MAPK and AKT/mTOR signaling (75, 76). Therefore, the synergistic cytotoxicity of the combination of barasertib plus melatonin towards leukemia lymphocytes could be explained by amplification of the effects of both substances on both molecular targets – aurora B kinase and mTOR.

In conclusion, there are many data regarding sensitization of cancer cells to conventional anticancer drugs (such as doxorubicin, bleomycin, cisplatin) by melatonin (34, 46, 47, 53-57), but only a limited number of studies have described the effect of melatonin on the cytotoxicity of new-generation anticancer drugs (recently approved for clinical use or still in clinical trials) (75-77). There are no data about the effect of melatonin on the efficiency of new-generation anticancer drugs described in the present study: AZD7762 (a selective inhibitor of checkpoint kinases), ABT-737 (a selective inhibitor of BCL2 proteins), barasertib (a selective inhibitor of aurora B kinase), everolimus (mTOR inhibitor), lonafarnib (a farnesyltransferase inhibitor), MG132, MLN-2238 and bortezomib (proteasome inhibitors), and palbociclib (a selective inhibitor of cyclin-dependent kinases). To our knowledge, our study is the first to report synergistic cytotoxicity of melatonin in combination with everolimus or barasertib, which is specific for cancer cells and particularly for Jurkat acute lymphoblastic leukemia cells. Both combinations are harmless for normal lymphocytes. The data suggest that melatonin is a promising supplementary component for anticancer therapy, allowing reduction of the therapeutic doses of barasertib and everolimus, minimizing their side-effects. This statement can also be made for all investigated anticancer drugs whose cytotoxicity increased by combining them with melatonin.

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