Melatonin potentiates cisplatin-induced apoptosis and cell cycle arrest in human lung adenocarcinoma cells

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Abstract

Objectives: Melatonin produces anti-cancer effects via several mechanisms, including by induction of apoptosis. In this way, it has been shown to be of use, in combination with chemotherapeutic drugs, for cancer treatment. The study described here has evaluated effects of melatonin on cytotoxicity, apoptosis and cell cycle arrest induced with the chemotherapeutic agent cisplatin, in human lung adenocarcinoma cisplatin-sensitive cell line (SK-LU-1), which previously had only limit data.

Materials and methods: Cells of the SK-LU-1 line were treated with melatonin alone at 1–5 mM concentration or cisplatin alone 10–200 μ M, for 48 h in culture. Cytotoxicity was measured by MTT reduction assay. Apoptosis induction was detected by annexin V/PI staining using flow cytometric analysis and DAPI nuclear staining. Change in mitochondrial membrane potential ($\Delta\Psi$ m) was quantified using DiOC6(3) reagent and activities of caspases-3/7 were also investigated. DNA fractions were measured using propidium iodide (PI) staining.

Results: Melatonin or cisplatin alone had 50% (IC_{50}) cytotoxicity at 5 mM or 34 μ M concentrations respectively. Combination of 1 or 2 mM melatonin and cisplatin significantly augmented cytotoxicity of cisplatin by reducing its IC_{50} to 11 and 4 μ M, respectively. Consistently, combined treatment increased population of apoptotic cells by elevating mitochondrial membrane depolarization, activating caspases-3/7 and inducing cell cycle

arrest in the S phase, compared to treatment with cisplatin alone.

Conclusion: These data demonstrate that melatonin enhanced cisplatin-induced cytotoxicity and apoptosis in SK-LU-1 lung cancer cells. SK-LU-1 cell population growth inhibition was mediated by cell cycle arrest in the S phase. These findings suggest that melatonin has the potential to be used for NSCLC treatment in combination with a chemotherapeutic agent such as cisplatin.

Introduction

Lung cancer is one of the most common malignancies and is the leading cause of cancer death worldwide (1). Sixty percent of lung tumours are non-small cell lung cancer (NSCLC) adenocarcinomas (2); in general, it has only a 16% 5-year relative survival rate, for all stages (1). Median time to progression is 4.5–23.7 months (2), indicating that even early forms of treatment do not completely control remission of the tumour. Surgery to remove tumours is an option for early stage NSCLC, often along with other modalities such as radio- and chemotherapies. NSCLC patients are commonly treated using platinum-based chemotherapy (cisplatin and carboplatin) or a taxane-containing regimen (paclitaxel and docetaxel) (3). These however, produce undesirable side effects on normal cells or tissues, leading to limits in their use as treatment for cancer. Thus, many recent studies report that chemotherapy is enhanced when in combination with appropriate supplements to overcome deleterious problems of chemotherapeutic agents (4,5).

Melatonin, *N*-acetyl-5-methoxytryptamine, is an indoleamine molecule produced primarily by the pineal gland and other organs. Its metabolic purpose is to control circadian rhythms (6) and is frequently used to

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treat insomnia, jet-lag (7), migraine (8) and headaches (9). A number of studies has reported it to have anti-oxidant (10,11), anti-inflammatory (12), immunomodulation (13,14) and anti-cancer (14,15) effects. Anti-cancer activity of melatonin has been reported against various neoplasms, including breast cancer (16,17), hepatocellular carcinoma (18–21), colon cancer (22), leukaemia (23–25) and pancreatic carcinoma (26). Melatonin exerts anti-proliferative effects on breast cancer cell population growth (16) and inhibition of hepatocellular carcinoma cell expansion by arresting cell cycles in the S phase (19). Induction of apoptotic cell death by melatonin has also been found in breast cancer (17) and hepatocellular carcinoma cell lines (20). Apoptosis is a well known mechanism of programmed cell death that does not injure normal neighbouring cells and avoids local inflammation (27). Activation of caspases by melatonin has also been reported in numbers of cancer cell lines such as those of human colon, leukemia, breast, pancreas and hepatocellular carcinoma (17,20,22,24–26), the caspase cascade having a major role in apoptosis induction pathways. Apoptotic induction has also been shown by depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) in melatonin-treated leukaemia cells (25). A recent report of Kim *et al.* revealed that melatonin treatment enhanced apoptosis induced by genotoxic stress in the cell cycle, and in apoptosis regulator 2 (CCAR2)-deficient NSCLC (A549 and A427) cell lines, after UV irradiation (21). *In-vitro* anti-cancer effects of melatonin have also been confirmed in animal studies. It has inhibited tumour cell growth in a chemically induced ovarian carcinoma rat model (28) and also in breast cancer xenografts in nude mice (29). Furthermore, many studies have reported melatonin to be a potent therapeutic agent in combination with chemotherapeutic drugs.

Combination studies of melatonin and chemotherapy against cancer cells have previously been reported to enhance chemotherapy induced-apoptosis in cancer cells, such as cisplatin-treated SK-OV-3 human ovarian cancer cells (30), doxorubicin-treated HepG2 and Bel-7402 human hepatoma cells (31), SK-N-MC human Ewing sarcoma cell line (32) and AR42J rat pancreatic tumour cell line (33). Little is known, however, regarding combination effects of melatonin and chemotherapeutic agents, in lung cancer.

The hypothesis of this study thus is that melatonin might play a role in increasing the response of lung cancer cells to cisplatin treatment. We investigated anti-cancer effects of combined treatment of cisplatin and melatonin on the SK-LU-1 NSCLC cell line. As cisplatin is a first line treatment for NSCLC in many countries (34), cisplatin-sensitive NSCLC grade III (SK-LU-1) cell line was used in the investigation. The combined effect of cisplatin and melatonin on SK-LU-1 cell death

and apoptosis were first evaluated, then caspases-3/7, $\Delta\Psi_m$ and cell cycle arrest were elucidated.

Material and methods

Cell culture and reagents

Human lung adenocarcinoma cisplatin-sensitive cell line SK-LU-1, was purchased from Cell Lines Service – CLS (Eppelheim, Germany) and maintained at 37 °C in 5% CO₂ atmosphere. Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen Life Technologies, Barcelona, Spain) supplemented with 10% foetal bovine serum and 100 U/ml penicillin and 100 µg/ml streptomycin was used in cultures. Cisplatin was from Boryung Pharmaceutical (Kyunggi-do, Korea) and melatonin (GMP) was manufactured by Huanggang Saikang Pharmaceutical Co. Ltd., Hubei, China (purity confirmed at >99.4% by DSC and HPLC). MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) was purchased from Amresco LLC (Solon, OH, USA). 3,3'-dihexyloxacarbocyanine Iodide (DiOC6(3)) and propidium iodide (PI) was from Sigma-Aldrich (St. Louis, MO, USA). 4'-6-diamidino-2-phenylindole (DAPI) was purchased from Sigma-Aldrich Chemie (GmbH, Germany). Biological grade cell culture reagents were purchased from appropriate commercially available suppliers. Melatonin was prepared by dissolving it in dimethyl sulphoxide (DMSO; Lab-Scan, Analytical Science, Dublin, Ireland) and pre-incubated for 2 h before cisplatin. Less than 1% v/v DMSO (in culture medium) was used, causing less than 10% cytotoxicity compared to untreated control cells.

Cytotoxicity detection by MTT reduction assay

Viable cells reduce MTT to formazan crystals which can be detected by colorimetric methods. Briefly, 5×10^3 cells were seeded in 96-well plates and left for 24 h; then they were treated with melatonin at specified concentrations. MTT solution (20 µl = 5 mg/ml) was added after 21 h treatment. MTT solution was removed and formazan was lysed with DMSO; thereafter it was measured using a microplate reader (Sunrise, Tecan Group, Mannedorf, Switzerland) at 555 nm with 650 nm reference wavelength. Finally, % cytotoxicity was calculated in comparison with the untreated group. IC₅₀ value was determined from cytotoxicity (%) versus concentration curve.

Evaluation of combined drug effects

For evaluating combined cytotoxic effects of melatonin and cisplatin, data from MTT assays were analyzed.

Combination ratio (CR) was calculated from ratio of estimated, and exact cytotoxicity. Estimated cytotoxicity (Es) was calculated from percentage of cytotoxicity of treatment alone (IC_{50} of cisplatin + IC_{50} of melatonin). Cytotoxicity effects of combined cisplatin and melatonin is represented as exact cytotoxicity (Ex). CR was then calculated from Exact cytotoxicity/Estimated cytotoxicity (Ex/Es) at each concentration of cisplatin. Combination effect was defined by CR value as follows: $CR > 1$ (synergistic effect), $CR = 1$ (additive effect) and $CR < 1$ (antagonistic effect).

Detection of apoptosis by annexin V/PI staining

Membrane phospholipid phosphatidylserines (PS) are exposed by apoptotic cells during the apoptotic process. PS translocates from the inner to the outer leaflet of the plasmamembrane, and it can bind to annexin V. Meanwhile, necrotic cells with internal and external membrane disruption can be stained with PI (35). This method is used by the manufacturer of the annexin V-FITC apoptosis detection kit (Cat no. 88-8005-74, eBioscience, San Diego, CA, USA). After completing melatonin treatment, cells were washed in phosphate buffered saline (PBS) and suspended in $1 \times$ binding buffer (HEPES-buffered saline supplemented with 25 mM $CaCl_2$). Then, 5 μ L annexin V-FITC and 5 μ L PI were added (15 min each), in the dark at room temperature. Once the incubation period was complete, cells were analyzed by flow cytometry (BD FACSCanto II, BD Biosciences, San Jose, CA, USA) within 1 h after staining.

DNA staining by DAPI

Apoptotic characteristics were revealed by DAPI staining. Intact nuclei within cells were stained and observed by the DAPI method of Yu *et al.* with modifications (36). After treatment with melatonin, cells were washed once in PBS, fixed in methanol for 10 min, then stained in 1 μ g/ml DAPI for 30 min. Results were expressed with complementary nuclear morphological observations gathered using fluorescence microscopy.

Determination of mitochondrial membrane potential

Changes in mitochondrial transmembrane potential ($\Delta\Psi_m$) during apoptosis was quantified using the cationic lipophilic dye DiOC6(3). DiOC6(3) (10 nM) was added to PBS-resuspended cells for 20 min. Then the dye was aspirated and cells were resuspended in PBS and assayed by flow cytometry (37).

Detection of caspase activity

Activity of caspases-3/7 was measured by colorimetry using caspase-glo 3/7 assay kit (Cat no. G8090; Promega, Madison, WI, USA) according to the manufacturer's instructions. After completing melatonin treatment, cells were washed in phosphate buffer saline (PBS) and suspended in $1 \times$ binding buffer. Briefly, 2×10^4 SK-LU-1 cells were seeded in 96-well plates and left for 24 h, then treated with melatonin. Lysates of SK-LU-1 cells were then mixed with equilibrated caspase-Glo 3/7 reagents for 1 h at room temperature. Luminescence was measured using an AB-2270 luminometer (Bio-Instrument ATTO, Tokyo, Japan).

Cell cycle phase determination using PI staining

Cell cycle distribution (G_0/G_1 , S and G_2/M) was determined by flow cytometry based on DNA content measurement of nuclei, stained with PI (38). After the incubation period, cells were trypsinized, then washed in PBS and stained with PI for cell cycle analysis. They were then washed in PBS and fixed in cold ethanol (70%) overnight at 4 °C. Next, cells were incubated in 1 ml 50 μ g/ml PI and 50 μ g/ml RNase A, for 1 h in the dark. DNA content was analyzed using flow cytometry. Relative proportions of G_0/G_1 , S and G_2/M cells were calculated.

Data analysis

Data were calculated as means ($\pm$ SD) and statistical comparisons were performed using one-way ANOVA with post hoc testing (multiple comparison) (SPSS; SPSS Inc., Chicago, IL, USA). Statistical significance within the 95% confidence interval was accepted when $P < 0.05$.

Results

Cytotoxic effect of melatonin in combination with cisplatin on SK-LU-1 cells

MTT reduction assay was performed on the cells to investigate whether melatonin influenced cytotoxicity of cisplatin. Melatonin treatment alone had a significantly cytotoxic effect at concentrations of 1, 2 and 5 mM (5.9 ± 0.7 to $59.0 \pm 2.1\%$, $IC_{50} = 5.0 \pm 0.1$ mM), compared to untreated controls (Fig. 1a). Cisplatin in concentrations from 10 to 200 μ M showed significantly higher cell death 20.7 ± 3.0 to $91.1 \pm 0.6\%$ ($IC_{50} = 33.9 \pm 1.3$ μ M), compared to controls (Fig. 1b). Melatonin at 1 mM (slightly higher cytotoxicity) and

Figure 1. Effect of melatonin (a), cisplatin (b) or the combination of melatonin with cisplatin (c) on cytotoxicity of SK-LU-1 for 48 h, evaluated by MTT reduction assay. Chemical structure and cytotoxicity of melatonin ($C_{13}H_{16}N_2O_2$, molecular weight (MW) = 232.28) and cisplatin ($Cl_2(NH_3)_2Pt$, MW = 300.04) are shown in dose dependent manner in (a) and (b), respectively. (c) The cytotoxicity of the combination of 1 or 2 mM melatonin and cisplatin (10–200 μ M) are also shown in SK-LU-1 cells. (d) Morphological changes of SK-LU-1 cells were observed under inverted microscopy (20 $\times$ magnification) after treatment with melatonin or cisplatin (50 μ M) or combination treatment for 48 h. Cytotoxicity is expressed as a percentage versus controls, cultured without any treatment. Five samples were averaged for each group. Error bars show SD of five samples. * P < 0.05 versus untreated cells, ^a P < 0.05 versus cisplatin treated alone.

2 mM (markedly higher cytotoxicity), causing less than 50% cytotoxicity, were selected for combination with various concentrations of cisplatin for combination cytotoxicity, apoptosis and $\Delta\Psi_m$. To confirm apoptotic induction mechanisms, 50 μ M cisplatin concentration causing in the order of 50% cytotoxicity, was used for DAPI staining, caspase activity and cell cycle analysis.

Importantly, treatment with the combination of melatonin and cisplatin showed significantly higher cytotoxicity compared to cisplatin alone, in a concentration-dependent manner (Fig. 1c). In the drug combination, 1 and 2 mM melatonin had the potential to reduce IC_{50} concentration of cisplatin alone from 50 μ M to 10.8 ± 0.4 and 3.8 ± 0.4 μ M, respectively in the SK-LU-1 cells. Results were confirmed by morphological observations (Fig. 1d).

Combination of cisplatin and melatonin was evaluated by combination ratio (CR) based on cytotoxicity from calculations (estimated cytotoxicity; Es) and experimental (exact cytotoxicity; Ex) data (Table 1). Combination ratio (CR) was calculated from the ratio of Es/Ex at each concentration of cisplatin. As expected, CR was >1 (1.1–1.7) in combination with cisplatin and 1 mM of melatonin at different concentrations of cisplatin (Table 1).

Enhancement of apoptosis by melatonin in combination with cisplatin

To elucidate further whether combination of melatonin and cisplatin in cell death was associated with apoptosis, annexin V/PI and DAPI staining assays were used (Figs 2,3). Percentage of annexin V/PI stained cells in

Table 1. Cytotoxicity of melatonin, cisplatin and the estimated and exact cytotoxicity values with a combination of melatonin and cisplatin in SK-LU-1 cells

Treatment	%Cytotoxicity				
Melatonin 1 mM	5.9 ± 0.7				
Melatonin 2 mM	40.6 ± 0.2				
Cisplatin (μM)	10	25	50	100	200
Combination treatment of cisplatin and 1 mM melatonin	20.7 ± 3.0	45.1 ± 2.1	59.4 ± 1.6	73.1 ± 0.2	91.1 ± 0.6
Estimated cytotoxicity (Es) ^a	26.6	51.0	65.3	79.0	97.0
Exact cytotoxicity (Ex) ^b	44.5 ± 1.0	71.4 ± 0.4	81.8 ± 1.5	91.8 ± 2.0	105.6 ± 0.5
Ex/Es ratio ^c (combination ratio: CR)	1.7	1.4	1.3	1.2	1.1
	Synergistic				
Combination treatment of cisplatin and 2 mM melatonin	61.2	85.7	99.9	100.0	100.0
Estimated cytotoxicity (Es) ^a	60.9 ± 1.8	86.6 ± 2.0	93.7 ± 1.1	99.9 ± 0.4	111.7 ± 0.1
Exact cytotoxicity (Ex) ^b	1.0	1.0	0.9	1.0	1.1
Ex/Es ratio ^c (combination ratio: CR)	Addition				

^aEstimated cytotoxicity (Es) = calculated cytotoxicity effect (IC₅₀ of cisplatin + IC₅₀ of MLT).

^bExact cytotoxicity (Ex) = the cytotoxicity effect of combined cisplatin and melatonin from the experiment.

^cEx/Es ratio or combination ratio (CR) = Exact cytotoxicity/Estimated cytotoxicity.

the combination treatment (Fig. 2e,f) was compared to that of each treatment alone (Fig. 2b–d). Percentage of annexin V/PI stained cells in each condition was calculated and is shown in Fig. 2g. Results indicate that there were more annexin V stained cells after combinatorial melatonin treatment (1 or 2 mM) and cisplatin (10–200 μM) treatment, in a dose-dependent manner (23.7 ± 3.3 to 47.9 ± 4.4% and 30.8 ± 6.6 to 73.0 ± 4.2% for 1 and 2 mM melatonin, respectively) than cisplatin treatment alone (Fig. 2g). Results reveal that cisplatin combination with melatonin (both 1 and 2 mM) increased cytotoxicity to the cells by apoptotic induction, supported by linear correlation between cytotoxicity and apoptotic induction (Fig. 2h).

Results were found to be equal by cell morphology analyses after staining with DAPI (Fig. 3). Consistently, they provide evidence confirming the observation that number of apoptotic cells, nuclear condensation, and fragmentation, were more observable after combination treatment of melatonin and cisplatin (Fig. 3e,f) than cisplatin treatment alone (Fig. 3b–d).

Induction of loss of ΔΨ_m by melatonin in combination with cisplatin

To demonstrate mechanisms underlying apoptotic induction in SK-LU-1 cells, the mitochondrial apoptotic pathway was investigated. Since loss of ΔΨ leads to apoptosis via mitochondrial pathways, levels of ΔΨ_m were determined. Results from flow cytometric analysis indicated that cisplatin increased loss of ΔΨ_m in a concentration-dependent manner. Loss of ΔΨ_m was significantly higher

by 48 h after combined treatment of melatonin and cisplatin compared to cisplatin alone (Fig. 4a). Results indicate that cisplatin alone or cisplatin combined with melatonin (both 1 and 2 mM) increased loss of ΔΨ_m and apoptotic induction, supported by linear correlation between cytotoxicity or apoptotic induction and ΔΨ_m (Fig. 4b).

Apoptotic induction by melatonin in combination with cisplatin by caspase activation

To demonstrate mechanisms underlying apoptotic induction, activity of apoptosis-related proteins in treated cells was investigated. After treatment with cisplatin (50 μM) for 48 h, activity of caspases-3/7 was remarkably higher compared to untreated cells, meanwhile melatonin alone resulted in no significant change. Interestingly, combination of melatonin treatment enhanced cisplatin-increased caspase-3/7 activity compared to the cisplatin control (Fig. 5). These results indicate that combination of cisplatin and melatonin enhanced apoptosis through caspase activation.

Cell population growth inhibition by S phase arrest with melatonin in combination with cisplatin

To evaluate effects of cisplatin and melatonin on SK-LU-1 cell cycle phases, DNA content was measured by flow cytometry (Fig. 6). Fractions of cells in S and G₂/M phases significantly increased, together with significant reduction in G₀/G₁ phases, after 48 h cisplatin treatment. Interestingly, 1 and 2 mM melatonin

Figure 2. Effect of melatonin, cisplatin and the combination of melatonin with cisplatin on the induction of apoptosis of SK-LU-1.

Flow cytometry analyses of SK-LU-1 cells using double staining with annexin V (Annexin V, horizontal line) and propidium iodide (PI, vertical line); (a) untreated group; melatonin treated group at (b) 1 mM and (c) 2 mM; (d) cisplatin treated group (50 μM); (e) combination treatment of 1 mM melatonin and cisplatin (50 μM) and (f) 2 mM melatonin and cisplatin (50 μM). Flow cytometry apoptotic results are shown in four subpopulations which indicate: early apoptotic cells (lower right), late apoptotic cells (upper right), normal cells (lower left) and necrotic cells (upper left). (g) The level of SK-LU-1 cell apoptosis expressed as the percentage of apoptotic cells are depicted in the graph (mean ± SD of at least three samples). (h) Correlation graphs of apoptotic cells and cytotoxicity of cisplatin treated SK-LU-1 alone and combination treatment are shown. Error bars show SD of three samples. $^aP < 0.05$ versus cisplatin treated alone.

treatment further increased cisplatin-induced S phase arrest from $17.8 \pm 1.4\%$ to 20.1 ± 0.4 and $21.0 \pm 1.8\%$, respectively. These results indicate that the combined treatment inhibited S–G₂/M transition in the cells, leading to cell population growth arrest.

Discussion

An important limitation of chemotherapy treatment for NSCLC is from undesirable or severe side effects leading to limited therapeutic efficacy. Recent studies have reported that combined chemotherapy and supplements are useful for improving therapeutic efficacy to overcome the problems caused by chemotherapeutic agents (4,5). Wang *et al.* reported that combination of gambo-

gic acid with cisplatin displayed synergism for causing death of A549 and NCI-H460 human NSCLC cell lines (4). Elkady *et al.* reported that crude alkaloid extract of *Rhazya stricta* (a medicinal plant used in traditional oriental medicine), increased cisplatin-induced apoptosis and inhibition of cell proliferation (5) in A549 cancer cells.

Anti-cancer effects of melatonin have been reported in many types of cancer (15–17,20,23), including that of the lung cancer (14,39,40). Our study has revealed that 1, 2 and 5 mM melatonin treatment for 48 h had a cytotoxic effect on SK-LU-1 cisplatin-sensitive lung cancer cells ($IC_{50} = 5.0$ mM). The results support our recent report that direct toxicity of melatonin on these cells was exhibited at high concentrations (14). This confirms recent studies on cisplatin non-sensitive A549 lung

Figure 3. Apoptotic bodies of SK-LU-1 after treatment with melatonin, cisplatin and the combination of melatonin and cisplatin detected by DAPI staining; (a) untreated group; melatonin treated group at (b) 1 mM and (c) 2 mM; (d) cisplatin treated group (50 μ M); (e) combination treatment of 1 mM melatonin and cisplatin (50 μ M) and (f) 2 mM melatonin and cisplatin (50 μ M). Apoptotic bodies were observed under inverted fluorescence microscopy (20 $\times$ magnification).

Figure 4. Effect of melatonin, cisplatin and the combination of melatonin with cisplatin on the mitochondrial membrane potential ($\Delta\Psi_m$) of SK-LU-1. Flow cytometry analyses of loss of $\Delta\Psi_m$ SK-LU-1 cells using DiOC6(3) staining. (a) The level of loss of $\Delta\Psi_m$ SK-LU-1 cells expressed as a bar graph (mean $\pm$ SD of duplicate experiments). (b) Correlation graphs of the loss of $\Delta\Psi_m$ cells and apoptotic cells or cytotoxicity of cisplatin treated SK-LU-1 alone or the combination treatment with melatonin (1 or 2 mM) are shown. $^aP < 0.05$ versus cisplatin treated alone.

Figure 5. The activity of caspase-3/7 in SK-LU-1 cells after treatment with melatonin, cisplatin and the combination of melatonin with cisplatin. Caspases activity was assessed using caspase-Glo assay. The incremental increase of caspases activity is expressed as a percent versus control, untreated group. Error bars show SD of at least two experiments ($n = 6$). * $P < 0.05$ versus untreated cells, $^{**}P < 0.05$ versus cisplatin treated alone.

cancer cell line, that melatonin treatment for 72 h at concentrations of 0.1–5 mM increased their cell death ($IC_{50} = 1.9$ mM) (39). However, results are still controversial, as Fic *et al.* did not find any change in number of A549 cells after exposure to 0.1 and 1 mM melatonin for 24 h (40). This indicates a variety of possible responses of cancer cell lines in different single melatonin treatment conditions (39,40).

Combination of melatonin and more conventional chemotherapy against cancer cells has been reported to enhance chemotherapy-induced apoptosis. Kim *et al.* reported that melatonin increased cisplatin-induced apoptosis *via* a caspase-dependent pathway in SK-OV-3 ovarian cancer cells (30). Melatonin with chemotherapeutic agents has also been reported to augment apoptotic cells in SK-N-MC human Ewing sarcoma cell line, by activation of caspases (32). Consistently, combined

Figure 6. Alterations of the cell cycle measured in the SK-LU-1 cells after treatment with melatonin, cisplatin and the combination of melatonin with cisplatin; duplicate experiments were performed while the DNA fraction profile from one experiment is shown; (a) untreated group; melatonin treated group at (b) 1 mM and (c) 2 mM; (d) cisplatin treated group (50 μ M); (e) combination treatment of 1 mM melatonin and cisplatin (50 μ M) and (f) 2 mM melatonin and cisplatin (50 μ M). (g) Percentage of cell numbers of each cell cycle phase in each group of treatment are shown as a bar graph (mean $\pm$ SD of duplicate experiments). * $P < 0.05$ versus untreated cells, $^{**}P < 0.05$ versus cisplatin treated alone.

treatment has been shown to elevate mitochondrial membrane depolarization in a AR42J rat pancreatic tumour cell line leading to apoptotic induction (33). Moreover, in previous studies on combination of melatonin with chemotherapeutic drugs, melatonin synergistically increased doxorubicin-induced cytotoxicity in A549 cells (40). Mechanisms responsible for combined effects of melatonin on chemotherapy-induced cell death in lung cancer cells, however, still remain unclear.

In this report, results indicate that melatonin significantly increased cytotoxicity of cisplatin-induced cell death. Melatonin indeed intensified effects of cisplatin on apoptotic induction by activating caspase activity and reducing $\Delta\Psi_m$. Furthermore, combined effects of cisplatin and melatonin were mediated by cell population growth inhibition *via* induced cell cycle arrest in the S phase.

Two major processes responsible for cell death are apoptosis and necrosis (27). In this study, annexin V/PI staining was used to detect apoptotic and necrotic cell death. Results of the combined effect of melatonin and cisplatin indicated higher apoptotic cell death than with cisplatin treatment alone. Moreover, more cells with condensed chromatin and nuclear pyknosis were detected by DAPI. Bright fluorescence of DAPI increased combination of melatonin and cisplatin, indicating that combined treatment induced more apoptotic bodies. Similar to these results, previous studies have shown that melatonin alone (20,41,42) or in combination with chemotherapeutic agents (30,32,33) induced apoptosis in several cancer cell types. Results here confirm the enhancing effect of melatonin on cisplatin-induced apoptosis in SK-LU-1 lung cancer cells.

Apoptotic induction *via* the mitochondrial pathway is an important mechanism initiated by chemotherapeutic agents (43). Depolarization of $\Delta\Psi_m$ leads to release of pro-apoptotic proteins, such as cytochrome c, from inner membranes of mitochondria, leading to activation of executioner caspase-3 to induce apoptosis (43). In this study, melatonin combined with cisplatin significantly reduced $\Delta\Psi_m$ and activated caspases-3/7. As has been reported previously, apoptotic induction can occur by melatonin activated through the caspase cascade, in various cancer cell lines such as of human colon cancer, leukaemia, breast cancer, pancreatic carcinoma and hepatocellular carcinoma (17,20,22,25,26) and decrease in $\Delta\Psi_m$ (25). Taken together, these findings are consistent with previous work on combined effects of melatonin and cisplatin in apoptotic cell death of SK-OV-3 human ovarian cancer (30) and AR42J rat pancreatic tumour cell lines (33) where melatonin increased apoptosis compared to treatment with cisplatin alone. Loss of $\Delta\Psi_m$ from cisplatin was also potentiated by melatonin

in AR42J cells (33). Consistently, cleavage of caspase-3 and poly-(ADP-ribose) polymerase (PARP) were enhanced by combined treatment of cisplatin and melatonin in SK-OV-3 (30).

DNA content analysis, showed by flow cytometry, that melatonin enhanced cisplatin-induced S phase cell cycle arrest. This result concurred with previous data indicating that S phase in HepG2 hepatocarcinoma cells was arrested by melatonin alone (19) or when combined with cisplatin (44). The result revealed that melatonin must have increased cisplatin effect on cell cycle arrest through inhibition of DNA synthesis (S phase arrest). Previous reports have shown that melatonin reduced mammalian target of rapamycin (mTOR) a protein involved in tumorigenesis and progression (45). Melatonin also has inhibited expression of DNA excision repair cross complementary 1 (ERCC1) protein, DNA repair mechanism, by returning damaged DNA to normal (46). Thus, it seems from our study that melatonin might enhance cisplatin's effect by inhibiting mTOR and ERCC1 expression.

In conclusion, this investigation has demonstrated that melatonin potentiated cisplatin-induced cytotoxicity and apoptotic cell death, and induced cell cycle arrest in SK-LU-1 cells. Combined treatment increased apoptotic induction by activation of caspases-3/7 and reduction in $\Delta\Psi_m$. These experiments indicated that melatonin enhanced cisplatin-induced apoptosis and prevented cell cycle progression. These findings might suggest a basis for guiding combinational treatment of melatonin and chemotherapeutic drugs for improving therapy efficiency for NSCLC. However, melatonin and cisplatin combination treatment in clinical trials must be performed before application of melatonin as the supporting agent for therapeutic use.

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Competing interests

The authors declare that there are no conflicts of interest.

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