Supplementation of melatonin protects human lymphocytes in vitro from the genotoxic activity of melphalan

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Melatonin (MLT) is a natural oncostatic factor of the human body as well as an antioxidant thus protects the nuclear DNA from oxidative damage. It also has the ability to reduce the side effects of various drugs when used as a combination therapy. The anti-neoplastic agent melphalan (MEL), which encompasses a number of side effects, is a strong alkylating agent and a potent inducer of sister chromatid exchanges (SCEs). The aim of the current in vitro study was to investigate the ability of MLT to reduce the genotoxic effect of MEL on normal human cultured peripheral lymphocytes. Cells were treated with both agents at various concentrations (MEL 100, 200 and 400 μM and MLT 330, 490 and 650 nM) and incubated for 72 h prior harvesting. The levels of cytostaticity, cytotoxicity and genotoxicity were qualitatively evaluated using the proliferation rate index, the mitotic index and the SCE methodology, respectively. Our results demonstrated the protective effect of MLT on cells treated with MEL in vitro. The greatest protective effect of MLT at 100 and 400 μM was illustrated against 330 nM of MEL in comparison with all other doses of MEL. These observations imply that MLT may be proved useful in reducing some of the toxic effects associated with certain classes of chemotherapeutic agents and other chemical and physical mutagens and carcinogens, acting both as an antioxidant–radical scavenger and a protective mechanism against cellular damage due to exposure to free radical-producing agents. It is essential to investigate substances with protective properties which are normally produced from the human body.

Introduction

Under diseased conditions, disturbance in the pro-oxidant–antioxidant balance leads to oxidative stress. Reactive oxygen species (ROS) in cells, in excess, are known to damage DNA resulting in signal transduction and gene expression to mutagenesis, transformation, mutagenesis and apoptosis (1–3). ROS are also produced by exogenous agents like radiation, chemicals, etc. To counter this, cells have acquired endogenous antioxidants, which scavenge ROS at different levels. Melatonin (MLT) is a potent anti-mutagenic agent against a variety of mutagens in vitro and in vivo (4–8). Its direct effects on the cell cycle, its abnormal fragmentation in tumour cells and influence metastasis by affecting the permeability of cellular membranes. It also stimulates glutathione (GSH) for the hydroxyl radicals (•OH) (6,7), as well as, a 15-fold more effective scavenger than the exogenous scavenger mannitol (21). Since MLT was found to act as a Ca2+-calmodulin antagonist, it is possible that it may have an anti-neoplastic action and hence, it may induce DNA fragmentation in tumour cells and influence metastasis by affecting the permeability of cellular membranes. It also stimulates GSH peroxidase (22), which catalyses the oxidation of GSH (reduced form) to its disulfide form GSSG (GSH, oxidized form) and simultaneously converts hydrogen peroxide (H2O2) to H2O and thus reduces the formation of the toxic hydroxyl radicals (•OH). Furthermore, it stimulates GSH reductase, which converts GSSG back to GSH, thereby reconstituting GSH (7). The free radical-scavenging efficiency of MLT along with its indirect antioxidant properties have been repeatedly documented by numerous independent investigators. Animals subjected to whole-body irradiation along with simultaneous MLT administration exhibited increased survival rates (5,9,10,23).

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Furthermore, numerous studies report the ability of MLT to reduce the side effect of various xenobiotics and furthermore enhance the efficacy of the drugs (24). Thus, its use as a combination therapy with genotoxic anti-neoplastic agents might be advantageous for non-cancerous cells in specific and for the adverse side effects of chemotherapy in general. Such agents that cause severe DNA damage are nitrogen mustards, which are bifunctional alkylating agents active against resting and rapidly dividing tumour cells. Melphalan (MEL), a bis(chloroethyl)amine with potent alkylating properties, is one of the most clinically useful nitrogen mustards (150 µg/kg/day per os for 7 days) used in the treatment of multiple myeloma, breast cancer, non-Hodgkin’s lymphoma, testicular seminoma, osteogenic sarcoma and non-resectable advanced ovarian cancer (25). The usual oral dose for multiple myeloma is 6–8 mg daily for a period of 4 days (usual intravenous dose is 15 mg/m² infused over 15–20 min), in combination with other agents. The dose is adjusted, as required, on the basis of blood counts measured at approximately weekly intervals. High-dose MEL (180–200 mg/m²) combined with bone marrow or peripheral blood stem cell reconstitution is a common treatment in myeloablative regimens (25). According to the published data, the concentrations of MEL used for in vitro studies in human lymphocyte cultures were 5, 10 and 25 µg/ml (26), 150 ng/ml (27) and 0.5–1 µM (28) and 52–420 mM in HT-29 human colon cancer cell line (29). The clinical toxicity of MEL is mostly haematological and is similar to that of other alkylating agents (25). It causes the distortion of the DNA helix, thus eliciting a remarkable induction of the sister chromatid exchange (SCE) levels. Besides that, studies have shown that the acute toxicity of alkylating agents has been linked with oxidative and nitrosative stress (30).

In this investigation, we determined MLT’s anti-genotoxic potential using SCEs induced by MEL as genotoxic end points in cultured human lymphocytes. We tested the effect of MLT, at various concentrations, on the damage exerted by MEL. MLT can initiate oxidative and nitrosative stress, leading to apoptosis and anti-tumour therapy on the one hand and chromosome instability and mutagenesis on the other. The antioxidant MLT, effectively prevents MEL-induced DNA damage, suggesting that MLT may have preventive or therapeutic effects in MEL-induced chromosome instability and genotoxicity. Presently, the protective effect of MLT was evaluated with the simultaneous determination of three cyto genetic parameters: (i) the levels of SCEs, (ii) the proliferation rate index (PRI) and (iii) the mitotic index (MI) in human lymphocyte cultures. The method of SCEs has been proposed as a very sensitive, simple and rapid method for detecting DNA damage induced by mutagens and/or carcinogens and anti-mutagenic agents and/or subsequent DNA repair, while its application is very useful for monitoring and improving chemotherapeutic techniques in vitro and in vivo. SCE methodology is considered a more sensitive method than chromosome aberrations (CAs) since induced DNA damage can be demonstrated by the formation of SCEs even at low concentrations of genotoxic agents. Furthermore, the other two indices PRI and MI are useful indicators of the cytostatic and cytotoxic properties of various agents, including chemotherapeutic agents (31).

Materials and methods

Chemicals

MLT, MEL, 5-bromo-2-deoxyuridine (BrdU), bis-benzamide and colcemid were obtained from Sigma (Sigma-Aldrich, St.Louis, MO, USA). All chemicals were dissolved in distilled water except MLT and MEL which were first diluted in absolute alcohol and then further diluted in distilled water. The final concentration of ethanol was <0.2% in all cell cultures. Previous reports have demonstrated that such low ethanol concentrations do not have an effect on SCE levels, something that was confirmed in the present study (results not shown) (32,33).

In vitro experiments

Heparinized blood samples were obtained from six healthy male and female individuals between 18 and 33 years old, none of whom was under any medication or was a smoker. Cultures of peripheral lymphocytes were prepared in universal containers by adding 11 drops of whole blood to 5 ml of chromosome medium B (Biochrom KG, Berlin, Germany). These were incubated at 37°C for 72 h. Cells were treated with MEL (100–400 µM final concentration) and/or MEL (330–650 nM final concentration) and were allowed to proliferate, in the presence of BrdU at a final concentration of 5 µg/ml. After 70 h, 0.5 µg/ml of colcemid was added for 2 h and at the end of the incubation period cultures were harvested. Cultures were maintained in the dark to prevent or minimize BrdU photolysis. Chromosome preparations were stained by a modified Fluorescence plus Giemsa technique (34).

Scoring was performed in a blind fashion. Cells on the first, second and third and subsequent mitotic divisions were counted. Mean SCE values were evaluated only in suitable second-division metaphases.

In order to establish the PRI, 300–450 cells were counted and the following formula was used: PRI = (M₁ + 2M₂ + 3M₃ + ⋯)/N, where Mᵢ is the percentage of cells in the first division, M₂ in the second and M₃ in the third and subsequent divisions, while N is the total number of cells counted, i.e. (M₁ + M₂ + M₃). In addition, Mls for 3000–4500 activated lymphocytes were determined for all cultures.

Statistical analysis

Evaluation of MI and PRI was based on the χ² test (35,36). To compare various treatments, logarithmization of SCEs was performed using the one-way analysis of variance and the Duncan test as far as pair-wise comparisons were concerned. Correlations between SCEs and Mls or SCEs and PRIs were also determined. Furthermore, the protective effect of MLT on cells exposed to MEL was evaluated by comparing the expected values (EVs) and the observed values (OVs). The EV of MEL was calculated using the formula: OVₘₑˡₑ = 0.69 SCEs per cell (23% reduction) and simultaneous elicitation of cell cycle delay and MI reduction compared to control (Table I). The combined treatment of 330 nM MEL with MLT (all combinations) elicited a statistically significant reduction in SCEs levels when it was compared with MEL treatment alone (P < 0.01). In more detail, the combined treatment of 100 µM MLT with 330 nM MEL reduced the SCE levels down to 12.72 ± 0.69 SCEs per cell (23% reduction) and simultaneous treatment of 330 nM MEL with 200 µM MLT reduced the SCE levels down to 11.36 ± 0.63 SCEs per cell (31% reduction), while when 400 µM MLT were co-administered with 330 nM
MEL, the SCE levels were reduced down to 8.13 ± 0.51 SCEs per cell (50% reduction) (Table I).

The ability of MLT to protect human lymphocytes from MEL was also investigated in higher MEL concentrations (490 and 650 nM) and the results are illustrated in Table II. As it was expected, these particular concentrations elevated SCE frequencies most significantly (490 nM MEL: 19.71 ± 1.35 SCEs per cell and 650 nM MEL: 26.13 ± 1.59 SCEs per cell) compared to control cultures (5.65 ± 0.41 SCEs per cell) (P < 0.01). As mentioned above, cells were also treated with 100, 200 and 400 μM MLT alone and in combination with 490 and 650 nM MEL. All MLT concentrations had no effect on SCE levels compared to control SCE levels (100 nM MEL: 8.13 ± 0.51 SCEs per cell) in these experiments too. When cells were treated with both agents (in all possible combinations), the protective effect of MLT on cells against MEL was confirmed. A total of 100 μM MLT when treated simultaneously with 490 and 650 nM MEL reduced SCE levels down to 16.12 ± 1.17 SCEs per cell (18% reduction) and 21.89 ± 1.44 SCEs per cell (16% reduction), respectively (P < 0.01), compared to the respective MEL treatments alone. The presence of 200 and 400 μM MLT in cultures treated with 490 and 650 nM MEL protected cells from the genotoxic properties of MEL as above (P < 0.01). Concurrent administration of 200 and 400 μM MLT with 490 nM MEL gave rise to 14.63 ± 1.05 SCEs per cell (26% reduction) and 13.57 ± 0.91 SCEs per cell (31% reduction), respectively, while simultaneous administration of 200 and 400 μM MLT with 650 nM MEL gave rise to 19.71 ± 1.21 SCEs per cell (25% reduction) and 15.44 ± 1.04 SCEs per cell (41% reduction), respectively. In Table II, a statistically significant negative correlation between SCEs and PRI was observed in cultures treated with 650 nM MEL alone or in combination with MLT (r = −0.989, P < 0.01). This suggests that SCEs rates can increase, while at the same time the cell cycle delays (cytostatic action).

### Table I. The damaging effect of MEL treatment alone and in combination with various concentrations of MLT, in normal human lymphocytes in vitro

<table>
<thead>
<tr>
<th>Agent (concentration)</th>
<th>Mean SCEs ± SEM (range of values)</th>
<th>PRI</th>
<th>MI (%red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.46 ± 0.44a (1–12)</td>
<td>2.34b</td>
<td>32.0c</td>
</tr>
<tr>
<td>MLT (100 μM)</td>
<td>5.95 ± 0.37b (1–13)</td>
<td>2.12</td>
<td>24.5d</td>
</tr>
<tr>
<td>MLT (200 μM)</td>
<td>5.22 ± 0.39 (1–12)</td>
<td>2.10</td>
<td>18.5e</td>
</tr>
<tr>
<td>MLT (400 μM)</td>
<td>4.72 ± 0.36 (1–14)</td>
<td>2.06</td>
<td>14.0f</td>
</tr>
<tr>
<td>MEL (330 nM)</td>
<td>16.43 ± 0.78 (6–28)</td>
<td>2.28g</td>
<td>29.0h</td>
</tr>
<tr>
<td>MEL (100 μM) + MEL</td>
<td>12.72 ± 0.69 (3–21); EV = 16.92</td>
<td>2.12</td>
<td>23.5i</td>
</tr>
<tr>
<td>MEL (200 μM) + MEL</td>
<td>11.36 ± 0.63 (5–20); EV = 16.19</td>
<td>2.15</td>
<td>22.5j</td>
</tr>
<tr>
<td>MEL (400 μM) + MEL</td>
<td>8.13 ± 0.51 (3–18); EV = 15.69</td>
<td>2.18</td>
<td>21.1k</td>
</tr>
</tbody>
</table>

The SCEs frequency was based on 45–60 second generation metaphases for each donor and each treatment and experiment; for PRI, 300–450 cells were counted and for MLT 3000–4500 activated lymphocytes, for each donor and each treatment for both indices in each experiment. The results were based on three experiments from three donors. PRI and MI comparisons were made by the x²-test. For SCE comparisons, logarithmic transformation of the data was performed using the one-way analysis of variance and the Duncan test. The EV for each combined treatment was estimated as described in Materials and Methods.

*P < 0.05 versus line 8 and P < 0.01 versus lines 5–7.

### Table II. The damaging effect of 490 and 650 nM of MEL treatment alone and in combination with various concentrations of MLT, in normal human lymphocytes in vitro

<table>
<thead>
<tr>
<th>Agent (concentration)</th>
<th>Mean SCEs ± SEM (range of values)</th>
<th>PRI</th>
<th>MI (%red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.65 ± 0.41a (1–12)</td>
<td>2.31b</td>
<td>31.5c</td>
</tr>
<tr>
<td>MLT (100 μM)</td>
<td>5.87 ± 0.55b (1–14)</td>
<td>2.32b</td>
<td>26.0d</td>
</tr>
<tr>
<td>MLT (200 μM)</td>
<td>5.05 ± 0.43 (1–10)</td>
<td>2.30b</td>
<td>25.5e</td>
</tr>
<tr>
<td>MLT (400 μM)</td>
<td>4.48 ± 0.39 (1–10)</td>
<td>2.27b</td>
<td>21.0f</td>
</tr>
<tr>
<td>MEL (490 nM)</td>
<td>19.71 ± 1.35g (9–35)</td>
<td>2.25g</td>
<td>28.0h</td>
</tr>
<tr>
<td>MEL (100 μM) + MEL</td>
<td>16.12 ± 1.17gh (6–25); EV = 19.93</td>
<td>2.28h</td>
<td>27.5i</td>
</tr>
<tr>
<td>MEL (200 μM) + MEL</td>
<td>14.63 ± 1.05 (6–22); EV = 19.11</td>
<td>2.22i</td>
<td>27.0j</td>
</tr>
<tr>
<td>MEL (400 μM) + MEL</td>
<td>13.57 ± 0.91 (5–20); EV = 18.54</td>
<td>1.98j</td>
<td>25.4k</td>
</tr>
<tr>
<td>MEL (650 nM)</td>
<td>26.13 ± 1.59l (14–39); EV = 26.35</td>
<td>1.95l</td>
<td>23.0m</td>
</tr>
<tr>
<td>MEL (650 nM) + MLT</td>
<td>21.89 ± 1.44 (8–30); EV = 25.53</td>
<td>1.97m</td>
<td>23.5n</td>
</tr>
<tr>
<td>MEL (200 μM) + MEL</td>
<td>19.71 ± 1.21k (7–27)</td>
<td>2.05</td>
<td>23.0o</td>
</tr>
<tr>
<td>MEL (400 μM) + MEL</td>
<td>15.44 ± 1.04 (5–26); EV = 24.96</td>
<td>2.10</td>
<td>18.0p</td>
</tr>
</tbody>
</table>

The SCEs frequency was based on 45–60 second generation metaphases for each donor and each treatment and experiment; for PRI, 300–450 cells were counted and for MI, 3000–4500 activated lymphocytes, for each donor and each treatment for both indices in each experiment. The results were based on 3 experiments from 3 donors. PRI and MI comparisons were made by the x²-test. For SCE comparisons logarithmic transformation of the data was performed using the one-way analysis of variance and the Duncan test. The EV for each combined treatment was estimated as described in Materials and Methods.

*P < 0.01 versus lines 5–12.

### Discussion

One of the most interesting properties of MLT is that it belongs to the natural oncostatic factors of the human body (7). In addition, there is a significant correlation between MLT and aging because the levels of the antioxidant decrease with age, and consequently if it is supplied, it can increase lifespan (36). From 1958 until recently, MLT was supplied experimentally to normal people and to cancer patients. According to the experimental data, many cancer patients responded to the supplementation of the antioxidant (37–41). A systematic review of 10 randomized controlled trials including 643 cancer patients in total illustrated that MLT reduced the risk of death at 1 year, while the effects were consistent across MLT dose and type of cancer (14).

MLT is capable of both directly scavenging radicals and radical-related reactants as well as stimulating antioxidative...
enzymes and hence it can affect the rate of growth and proliferation of cancer cells (42). It has also been found that it inhibits the uptake and metabolism of linoleic acid via receptor-mediated processes, indirectly thus inhibiting the action of the mitogenic signalling molecule 13-hydroxyoctadeca-decadienoic acid in rat hepatoma cells (43). Moreover, there is evidence that MLT inhibits telomerase activity, an enzyme that maintains the integrity and stability of chromosomal structure. Studies in MCF-7 human breast cancer cell line illustrated that this action of MLT results in the death of cancer cells (44). Furthermore, this antioxidant enhances the apoptosis of cancer cells by inhibiting the synthesis of endothelin-1, a potent vasoconstrictor peptide that has been implicated in the regulation of cancer growth by protecting cancer cells from apoptosis and promotion of angiogenesis in tumors (45).

Its multiple properties in vivo may be due to (i) its ability to act upon receptors located at the cell membrane, cytoplasm or nucleus; (ii) its action is independent of a receptor; (iii) its ability to activate multiple signal transduction pathways; (iv) its ability to regulate MLT receptor’s function and (v) its ability to interact with multiple receptor subtypes throughout the body (8).

In this study, MLT’s anti-genotoxic effect was tested against MEL, a well-known chemotherapeutic agent that belongs to the class of nitrogen mustards, which encompasses a number of side effects since it is also highly mutagenic and clastogenic (46–48). The cytogenetic method employed in order to determine the anti-mutagenic properties of this antioxidant was the SCEs. An increase in the levels of chromatid exchanges in metaphase chromosomes directly reflects DNA damage (caused by various agents) and consequently indicates the failure of pre-replicative repair mechanisms to achieve full recovery of the damaged site thus leading to double-strand breaks (49,50). We examined, presently, the capability of MLT to modulate the repair of oxidative and nitrosative lesions, considered as a major side effect of various anti-cancer drugs and nitrogen mustards in specific. MLT was found to act as an antioxidant–radical scavenger by suppressing MEL-induced genotoxic damage. According to our findings, MLT alone did not increase the levels of SCEs per cell in human lymphocytes cultures. MLT concentrations ≥750 µM were also studied, but these proved to be highly cytotoxic, thus leading to an insufficient number of nuclei and mitoses for our analysis (data not shown). A total of 750 µM MLT proved also to be genotoxic, something that was not observed in previous studies (18,33). The increased cytotoxicity noticed can be attributed to the antagonistic action of MLT against the Ca2+-calmodulin system that plays a role in stabilizing the microtubules of the mitotic spindle (7).

PRI reduction was inversely proportional to MLT concentration. This cytostatic action can be attributed to the inhibition of thymidine kinase by MLT. Since thymidine kinase is an essential enzyme for DNA synthesis, MLT could inhibit the entrance of the cells to S phase of the cell cycle (51,52). The fact that MI was also reduced as the concentration of MLT increased could be due to the latter’s capacity to antagonize the Ca2+-calmodulin system, as mentioned above. PRI and MI reduction have also been reported in the past in human blood lymphocytes cultured continuously for 72 h in the presence of ≥0.2 mM MLT (33). It was suggested that these observations could be associated with the inability of lymphocytes to fully respond to phytohaemagglutinin (PHA) stimulation when MLT is present at the initiation of the cell cultures, since MLT-treated lymphocytes prior PHA stimulation did not illustrate similar findings (33). Since pre-replicative DNA repair mechanisms are involved in the repair of ROS-induced damage, cell cycle delays may enable cells to repair such DNA damage for a longer period of time. Nevertheless, not all damage is repaired. When the cell enters S phase, post-replicative mechanisms take action. One of these is homologous recombination, in which studies have shown that SCEs play an important role. MEL can cause oxidative and nitrosative stress (30). Thus, that is probably why MLT exhibits a suppressive effect on MEL-induced SCEs. Our findings suggest that the optimum range of concentrations of MLT was 100–400 µM, where there was no apparent DNA damage. Previous studies have also demonstrated that these MLT concentrations do not induce SCE levels (18,33).

On the other hand, MEL alone elicited a significant increase in the levels of SCEs per cell. The combined treatments of MLT and MEL lead to a remarkably significant reduction of the SCEs levels, compared with the treatments of MEL alone. When MLT at concentrations ranging from 100–400 µM was simultaneously administered along with MEL significantly decreased the levels of SCEs, something that was noticeable in all MLT concentrations studied (330, 490 and 650 nM). This is presented in Figures 1 and 2. According to our findings MLT’s ability to suppress the genotoxic effects of MEL was dose dependent (Figure 1) (r = +0.900, P < 0.01). The protective ability of MLT in human lymphocytes against MEL was as follows: 100 µM MLT more effective in 330 > 490 > 650 nM MEL; 200 µM MLT more effective in 330 > 490 > 650 nM MEL and 400 µM MLT more effective in 330 > 650 > 490 nM MEL. In Figure 2, the incline between the two lines demonstrates the protective effect of MLT on cells against MEL (P < 0.01). Indicatively, MLT at a concentration of 400 µM alone with MEL at a concentration of 330 nM reduced SCEs levels by 50% in comparison with MEL alone, while 400 µM MLT co-administered with 490 nM MEL reduced the SCEs levels by 31%. A total of 400 µM MLT co-administered with 650 nM MEL led to a 41% reduction of SCEs levels. The negative correlation observed between SCE levels and PRI signifies that DNA damage is not only responsible for SCE induction but also for the reduction of the proliferation rate of...
The cells. Second-division metaphases with increased SCEs levels represent actively proliferating (living) cells that continue to proliferate, bearing the genotoxic damage induced by MEL.

The protection that MLT provides to cells is probably due to its capacity to enter the nucleus and interact with chromatin. It interacts with double-stranded DNA and possibly stabilizes it (4), while MEL as an alkylating agent causes distortion of the helix. Since SCEs represent double-strand breaks, that is why MLT reduces the damaging effect of MEL on lymphocytes (49). Other studies have reported the ability of MLT to activate RNA polymerase II and have suggested a role for MLT in regulating the transcription of particular genes (8). Furthermore, it appears to have a cytoplasmic action, too (4,8). Studies have also found that MLT and MEL have contrasting actions since MEL requires the reduction of GSH’s protein levels in order to act cytotoxically (53). MLT is a potent antioxidant, while MEL a pro-oxidant (30). Thus, MLT can suppress the genotoxic effects of MEL through various mechanisms. It could be possible that at low MEL concentrations MLT might exert its protective role by acting directly on the DNA, while in high concentrations might mostly act as an antioxidant.

The physiological levels of MLT in the blood are in the picomolar and low nanomolar range 10–250 pg/ml (54). However, it has been illustrated that in other body fluids and cells, physiological levels of MLT are not necessarily in equilibrium with those in the blood. It has also been suggested that MLT may accumulate in some cellular structures such as cell membrane, cytosol, nucleus and mitochondria (55,56). Tissue concentrations also may not depend exclusively and represented accurately by those in the blood (57). There is evidence that apart from cells of the pineal gland, high MLT concentrations are very likely to exist in other cells such as some bone marrow cells (58), cells of the gastrointestinal tract (59) and cells in tissues like human ovary, cerebrosplinal fluid and gallbladder (57,60–63) that possess the synthetic machinery to produce the indole (57). In the bile of mammals, the levels of MLT range between 1100 and 11 000 pg/ml and in the cerebrosplinal fluid of ewes, the concentration of MLT is ~20-fold higher than in the blood (60,61). Therefore, a physiological concentration of MLT may be different in reference to the compartment, where it is investigated (64). As mentioned in our manuscript, MEL is used in the treatment of multiple myeloma, breast cancer, non-Hodgkin’s lymphoma, testicular seminoma, osteogenic sarcoma and non-resectable advanced ovarian cancer. This means that the action of MEL is more intense in these target tissues. In our study, MLT’s protective effect on MEL-induced DNA damage was investigated in normal lymphocytes in vitro. In clinical use, MLT will attempt to overcome MEL-induced damage throughout the body but mostly in normal cells in MEL’s target tissues. Thus, in order to evaluate the magnitude of increase in MLT levels by external administration, MLT levels should be compared with the physiological MLT levels in such normal and pathological tissues. Furthermore, in 46 cancer patients with multiple myeloma plasma, MLT concentrations were significantly higher than healthy subjects, suggesting that this represents a response to an increased demand of the developing tumour due to an altered endocrine–metabolic balance or a compensatory mechanism to regulate neoplastic growth due to MLT’s anti-mitotic properties (65).

Since both cancer and normal cells have similar DNA repair mechanisms, they also share the ability to excise and repair various types of damage to DNA. Thus, the genotoxic properties of chemotherapeutics on normal cells reflect the level of desirable damage, induced by the same chemotherapeutics upon cancer cells (48). Studies have demonstrated that the effectiveness of potential anti-tumour agents in SCE induction in vitro and in vivo correlates positively with the in vivo tumour response to these agents (66,67). Nevertheless, it is necessary in order to ascertain MLT’s chemoprotective effect to study higher MEL concentrations in normal cells and furthermore test MLT in cancer cells in vitro and in vivo, on which our team is focusing at the moment.

MLT has been reported to possess protective properties for normal cells after exposure to chemotherapeutic agents (39–41,68,69). The international literature has provided indications and sometimes proofs that MLT can function protectively against chromosomal damage and be used as a supplement to chemotherapy, since it is not toxic, it can reduce the side effects of chemotherapy, and it can increase the efficacy of anti-neoplastics and other xenobiotics (24). It was demonstrated that cancer patients treated with MLT and radiotherapy lived longer than patients treated with radiation alone (68). The concurrent administration of MLT and tamoxifen in 14 women with metastatic breast cancer seemed to slow the progression of the disease (69), while in 40 patients with advanced malignant melanoma treated with up to 700 mg/day MLT dose, 6 had transient decreases in the size of some tumour masses (38). A study in the bone marrow of mice illustrated that MLT reduces cis-platinum induced genetic damage (70). Other studies have reported the protective effect of MLT on rat hepatocytes from chromium (VI)-induced DNA single-strand breaks in vitro (71) and on human and mouse lymphocytes in vitro from X-ray and γ-induced induced mutagenesis (10,72–74). Furthermore, MLT decreased hepatic DNA adduct formation caused by safrole in rats (4, 6) and inhibited the formation of CAs and sperm head anomalies in mice treated with cyclophosphamide, 1,2-dimethylhydrazine and N-nitrosomethylurea (75). Other studies have also reported that it significantly reduced nitrogen mustard (methylthrethamine/HN2) induced toxicity in the lungs (30) and lead- and delta-aminolevulinic acid-induced SCE frequencies in human lymphocytes in vitro (76). Vijayalaxmi et al. (73) reported that increased MLT concentration in the serum

![Fig. 2. This scatter diagram represents the effect of MEL treatments alone (330, 490 and 650 nM) and in combination with 400 μM of MLT on the induction of SCEs, in normal human lymphocytes. The linear regression lines were calculated by the least square method (x-axis: concentration of MEL (nM), y-axis: SCEs per cell, SE = standard error). I: y = 5.609 + 0.031 × x (SE = 0.91) and II: y = 5.159 + 0.015 × x (SE = 1.72).](https://academic.oup.com/mutage/article-abstract/23/5/347/1034754)
and/or leukocytes was positively correlated with decrease in primary DNA damage and increase in numbers of cells bearing reduced levels of DNA damage. It has been suggested that the concurrent administration of MLT along with chemotherapy or radiotherapy can compensate for the damage caused to blood cells, therefore enhancing the tolerance to the treatment (77).

Fischer et al. (78) investigated the protective effect of MLT in ultraviolet-induced DNA damage in leukocytes in vitro. According to their findings, 2 mM MLT (464 µg/ml) revealed the highest suppression of ROS in dose–response studies from 0.1 nM to 2 mM (79). In our study, MLT concentrations ranged between 11.5 and 174 µM. The reason that we used these concentrations was because at smaller doses tested (0.05, 0.5, 5 and 50 µM) MLT was ineffective (results not shown) and since 750 µM proved to be cytotoxic (results not shown), we had to restrict the concentrations of MLT between 100—and 400 µM. At these concentrations, we were able to observe the protective effect of MLT on MEL in a dose-dependent manner.

About 1000 research studies (50% of which were performed in vivo) document that MLT reduces free radical damage (57). MEL is an external parameter that when inserted into the body causes oxidative and nitrosative stress. This means that ROS and NOS due to the presence of MEL increase significantly. Would not be logical for MLT in order to protect cells from MEL-induced DNA damage to exist in higher than physiological concentrations? It is possible that cells in order to be protected against MEL-induced or oxidative-induced DNA damage in general may require such high MLT concentrations (78). Reiter et al. (57) claimed that the prospect of physiological MLT concentrations (or any antioxidant) to overcome severe oxidative stress conditions is naive. Under such extreme conditions, the severe extent of molecular damage and cellular death it attributed to the fact that all physiological antioxidants combined (vitamin E, vitamin C, MLT, GSH, antioxidative enzymes, etc.) are incapable of restoring order. Therefore, it was suggested that under such extreme conditions, any agent with antioxidative properties must be administered in pharmacological doses (57).

It is essential to investigate substances with protective properties which are normally produced from the human body. Many researchers believe that MLT is a potent genoprotector, anti-carcinogenic and anti-tumour compound (80). It has been suggested that it may play an important role in defending cells from DNA damage induced not only by oxidative mutagens but also by different alkylating agents (80). These results suggest that MLT may be proved useful in reducing some of the toxic effects associated with certain classes of chemotherapeutic agents. MLT has antioxidant properties at both physiological and pharmacological levels and it is prudent not be overlooked as a protective agent against oxidative damage (57). It is a molecule with intense past, significant present and hopeful future.

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References


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