

Review Article

Anticancer Effects and uses of Melatonin: A Review

Alibek K^{1,2,3}, Mektepbayeva D^{1*}, Irving S², Atinbayeva N², Zhaisanbayeva B², Mussurova S² and Mussakhan S²

¹National Laboratory Astana, Nazarbayev University, Kazakhstan

²Nazarbayev University Research and Innovation System (NURIS), Nazarbayev University, Kazakhstan

³National Research Center of Oncology and Transplantation, Kazakhstan

*Corresponding author: Mektepbayeva D, National Laboratory Astana, Nazarbayev University, 53 Kabanbay Batyr Avenue, Astana 010000, Kazakhstan

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Abstract

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is a naturally occurring hormone secreted by the pineal gland. Clinical evidence suggests that MLT may have a possible role in the treatment of cancer, where MLT presents many oncostatic properties in a wide variety of tumors, utilizing multiple and converging mechanisms. It is a potent anti-oxidative agent; its circadian rhythm-regulating properties are crucial for orchestrating patterns of hormone secretion, the imbalance of which is implicated in a wide range of hormone-dependent cancers of the reproductive organs. Recent advances in cancer treatment can offer therapeutic alternatives that could reduce the severity of unwanted side effects. Several observational studies have demonstrated a relationship between long-term disruption of circadian rhythm with decreased MLT secretion and increased cancer risk, whilst clinical evidence supports the possible benefits from MLT on the survival in patients with a range of cancers. This review will address some of the multiple anticancer properties of MLT, with a particular focus on the mechanisms counteracting tumor occurrence, growth, and development. Recent research into the oncostatic effects of MLT and the mechanisms of action explaining its efficiency for tumor regulation are summarized in this review and suggestions for the therapeutic use of MLT will be presented.

Keywords: Melatonin; Cancer treatment; Anti-oxidant; Anti-proliferative; Immunoenhancer

Abbreviations

Melatonin: N-acetyl-5-methoxytryptamine; NCI: National Cancer Institute; IL: Interleukin; 8-OH-G: 8-hydroxyguanine; •OH: Hydroxyl; LAN: Light-at-night; LDL: Lipoproteins; MCF-7: Michigan Cancer Foundation-7 (human breast cancer cells); MR-MT1 and MT2: Melatonin Receptors; ROS: Reactive Oxygen Species; AMK: N1-acetyl-5-methoxykynuramine; AFMK: N(1)-acetyl-N(2)-formyl-5-methoxykynuramine; NOS: Nitric Oxide Synthases; NO: Nitrogen Oxide; RARs: Retinoid Acid Receptors; NFκB: Nuclear Factor kappa-light-chain-enhancer of Activated B Cells; I-κB: Nuclear Factor of kappa Light Polypeptide Gene Enhancer in B-cells Inhibitor; RORα: RAR-related orphan receptor-α; CaM: Calmodulin-dependent Kinases; BER: Base-excision Repair; LH: Luteinizing Hormone; ER: Estrogen Receptor; ERα: Estrogen Receptors Alpha; FSH: Follicle-stimulating Hormone; ERE: Estrogen Response Element; cAMP: Cyclic Adenosine Monophosphate; MAPK: Mitogen-activated Protein Kinases; MEKs: Mitogen-Activated Protein Kinase Kinases; ERKs: Extracellular-signal-regulated Kinases; SEEM: Selective Estrogen Enzyme Modulators; SERM: Selective Estrogen Receptor Modulators; LA: Linoleic Acid; 13-HODE: 13-hydroxyoctadecadienoic Acid; 15-LOX-1: 15-lipoxygenase; MHCs: Major Histocompatibility Complexes; NK: Natural Killer cells; CD4: Cluster of Differentiation 4; Th: T-helper cells; DC: Dendritic Cells; IFN: Interferon; E2: 17-beta-estradiol; HepG2: Hepatocellular Carcinoma cell line; MG-63: Human Osteosarcoma cell line; HL-60: Human Myeloid cells; CDK: Cyclin-dependent Kinase; p53: Tumor Protein; p21: Cyclin-dependent Kinase Inhibitor 1; Jar cells: Human Choriocarcinoma; JEG-3: Human Placental Choriocarcinoma Cell Line; RHE: Acute Lymphoid Leukemia;

RAMOS: Burkitt Lymphoma; VEGFs: Vascular Endothelial Growth Factors; PI3K/AKT: Phosphatidylinositol-3-kinase; HIF-1: Hypoxia-induced Factor-1; DNMTs: DNA Methyltransferases; HATs: Histone Acetyltransferase; TIMP: Tissue Inhibitor of Metalloproteinase; BRCA1: Breast Cancer Type 1 Susceptibility Protein; p16: Cyclin-dependent Kinase Inhibitor 2A; GPC3: Glypican-3; EGR3: Early Growth Response Protein 3; POU4F2: POU Domain, Class 4, Transcription Factor 2; AP-1: Activator Protein 1; TNF-α: Tumor Necrosis Factor; COX-2: Cyclooxygenase-2; iNOS: Cytokine-inducible Nitric Oxide Synthase; MMPs: Matrix Metalloproteinases; NMR: Nuclear Melatonin Receptors; NR: Nuclear Receptor; PPARs: Peroxisome Proliferators-activated Receptors; RXR: Retinoid X Receptor; GR: Glucocorticoid; RZR: Retinoic Z Receptor

Introduction

All cancers, combined, comprise the second most prevalent cause of mortality worldwide. The development of procedures aimed at prevention and treatment to control cancer depends on the understanding of cancerous cells and the mechanisms through which they occur [1]. The National Cancer Institute (NCI) describes the most commonly used treatments as chemotherapy, radiation therapy, and immunotherapy, although transplantation, laser treatment, targeted therapy, and angiogenesis inhibitors are further options. Nevertheless, as cancer treatments and therapies advance, severe side effects increase [2]. This can be seen in weakness of the immune system, coronary heart disease, problems with reproduction, hair loss, vomiting, rectal bleeding, bladder irritation, chronic pain, and anaemia [2,3].

Melatonin (N-acetyl-5-methoxytryptamine, MLT) is a hormone

secreted by the pineal gland with serum levels peaking between 2AM and 5AM and has a first distribution lifetime of 2 minutes and a second metabolic half-life of 20 minutes [4]. Discovered in the late 1950s, anticancer effects of MLT research dates back to the 1970s, where a possible link between MLT inhibition and breast cancer was reported [5]. Further research has since shown this effect is likely the product of MLT-regulating tumor growth [4].

MLT has several therapeutic applications, such as antioxidative effects, where it has been used as treatment for ionizing radiation in Japan following the Fukushima Daiichi incident [6], as well as having a positive effect on the immune system, due to lymphocyte number control and inducing the production of a number of interleukins [7]. More recently, MLT has been shown to contribute to cancer prevention and has been trialled as a secondary drug in cancer treatment. Recent evidence suggests it can play a role in inhibiting the growth of cells in a number of cancers, including breast cancer, ovarian cancer, colon cancer, and cervical cancer [8].

Similarly, decreased levels of MLT have been linked to several kinds of cancer, neurodegenerative disorders, aging, and immunosenescence. For example, breast cancer cell proliferation is increased during the daytime when there are low MLT levels, compared to during the night when there are increased MLT levels and lower cell proliferation [9]. Therefore, factors that reduce nighttime MLT concentrations may be involved in the promotion of breast cancer. While many of these studies have used MCF-7 human breast cancer cells, similar results have been obtained with human Leiomyosarcoma [9] and rat hepatoma cells [10].

MLT studies in mice have established the boundaries of effective dosages for cancer patients. With prostate cancer xenograft mice, the concentration of 1 mg/1 kg administered as intraperitoneal injections resulted in statistically significant plasma levels of MLT (4 nM), which was sufficient for an anti-tumor effect [11]. Similarly, injections of MLT coupled with reduced light-at-night (LAN) conditions in athymic breast cancer mice (MDA-MB-231 cell line) at 40 mg/1 kg resulted in anti-tumor effects that corresponded with decreased rates of cell proliferation and neovascularization [12]. When applied to humans, the diagnosed stage of cancer is a key factor for recommended dosage; however, even small doses of MLT can have a significant impact to the results of the treatment [8]. In patients with pancreatic cancer, breast cancer, and progressive solid cancer, when MLT is administered alongside chemotherapy, it has been reported that there is an increase in sleep quality, reduced chemotherapy-induced side effects, disease stabilization, and increased survival rate [13-15].

The optimal dosage used in clinical trials varies between 20-40 mg, distributed throughout the day. Administration of MLT with tamoxifen has been found to regress metastatic breast cancer, and MLT combined with interleukin-2 has been shown to have an effect on tumor regression and disease stabilization. This has been found in lung carcinoma, liver carcinoma, bowel carcinoma, stomach carcinoma, pancreatic carcinoma and breast cancer [16]. Chemotherapy is the therapy of choice for many oncologists in the treatment of cancer, despite the destructive effect on the human organism. When used in conjunction with MLT, however, there is evidence of reduced toxicity and unpleasant side effects [17,18].

In this review, MLT-associated anticancer effects and their mechanisms will be discussed. Primarily, we focus on the relationship of anticancer therapy with antioxidative and anti-estrogenic properties, the activation of the immune system, the effect on metastasis, and epigenetic actions (Figure 1).

Anti-oxidative properties

Cancer is a proliferative disease where many tumors can develop following unrepaired damage to nuclear DNA, which is frequently the result of increased concentrations of highly reactive chemicals known as free radicals [19]. Free radicals are naturally produced in the body during the mitochondrial electron-transport chain step in respiration and are, generally, tightly regulated to prevent overproduction. Excessive numbers of free radicals can direct tumorigenesis-associated intracellular signalling [20]. Oxidative damage to lipids and proteins is an important physiological dysregulation step as the products of such reactions can be potentially carcinogenic: for instance, malondialdehyde, a major product of lipid peroxidation, is both mutagenic and carcinogenic in animal models [21]. The mutagenic properties of free radicals derive from their potential for interaction with all parts of the DNA molecule, oxidizing both nitric bases and the deoxyribose backbone. Thus, 8-hydroxyguanine (8-OH-G) formation, as a result of attacks from free hydroxyl radicals, is a major indicator of high levels of oxidative stress and considered to be one of the most crucial pre-mutagenic molecular lesions [22].

Unlike other antioxidative agents, MLT is able to permeate all morphophysiological barriers and reach therapeutically relevant concentration levels; it binds to target cells via two types of G protein-coupled MLT receptors (MR) - MT1 and MT2. Although a variety of cells express MT1 and MT2 (ranging from the epithelial lining of the gut to the cells of salivary glands, as well as lymphocytes), it is the cells of the hypothalamic suprachiasmatic nucleus and hypophyseal pars tuberalis that express MT1 at the highest density [23]. These cells are targets of MLT on the brain-endocrine axis. MT2 receptors are mostly expressed in the inner plexiform layer of the retina, which mediates the light stimulation responses in MLT production [24].

There are several antioxidative mechanisms of MLT, including single-electron transfers, pro-oxidant and antioxidant enzyme regulation, and mitochondrial metabolism control [25-27]. As MLT offers a range of both direct and indirect free radical neutralizing effects, it protects DNA from the mutagenic oxidative damage they inflict [28,29]. The high efficiency of MLT in reducing free radical-mediated mangling of DNA indicates that the indoleamine must be in the nucleus in sufficient concentrations to counteract the damaging effects of any radicals produced in the vicinity [30].

As an electron donor in single-electron transfer reactions, MLT acts as a direct scavenger of oxygen and nitrogen-based free radicals, as well as non-radical reactive oxygen species (ROS), such as hydrogen peroxide. The antioxidative mechanisms of MLT involve the indole ring binding of $\bullet\text{OH}$ (hydroxyl) at carbon 2 of the indole ring. For an antioxidant to protect the genome from destruction by $\bullet\text{OH}$, the protective agent must straddle the DNA. The most toxic and reactive radicals are estimated to travel only a few angstroms and have a half-life of only a few nanoseconds before interacting with a bystander molecule [30]. The immediate product of pyrrole ring cleavage is cyclic 3-hydroxymelatonin. In addition, N1-acetyl-

5-methoxykynuramine (AMK) and N(1)-acetyl-N(2)-formyl-5-methoxykynuramine (AFMK), utilize similar mechanisms to achieve antioxidative effects [31]. AMK, a protective metabolite formed during electron donation, is particularly relevant for mitochondrial metabolism as it acts to reduce electron leakage by maintaining membrane potential and aiding electron flux in the transport chain [25].

MLT acts to downregulate prooxidant enzymes such as lipoxygenases and nitric oxide synthases (NOS), which increase the levels of oxidative stress via increasing the production of ROS/RNS [25]. NOS produces nitrogen oxide (NO), a free radical capable of driving peroxynitrite synthesis due to the reactions between NO and the superoxide anion. Peroxynitrite is highly reactive and contributes to both lipid and protein peroxidation [26]. Lipoxygenases catalyze reactions of lipid peroxidation, resulting in enzyme-specific hydroperoxides acting as biomediators in signalling pathways. However, if lipid oxidation occurs at pathological levels, oxidative damage to low-density lipoproteins (LDL) is accumulated with concurrent production of ROS/RNS [32]. By directly downregulating the activity of both of these enzymes, MLT exercises its antioxidative properties. The exact mechanisms of downregulation are tissue specific; MLT does not suppress normal physiological activity of prooxidant enzymes, which makes it a preferential therapeutic agent. For example, NOS activity resulting in basal negligible NO radical production is crucial for cellular communication in neuronal and immunological networks. Moreover, the activities of lipoxygenases 5, 12 via possible interactions with retinoid acid receptors (RARs), which are related to oxidant generation, have not been substantially investigated [33].

A mechanism of oxidative stress regulation by MLT is utilizing the NF- κ B pathway: in the absence of oxidative stress, NF- κ B is found in the nucleus in the form bound to the inhibitory subunit I- κ B. When exposed to higher levels of ROS, I- κ B phosphorylation triggers NF- κ B translocation to the nucleus, which results in binding to the appropriate response elements of the genes encoding antioxidative enzymes, increasing their transcription levels [34]. I- κ B activity is upregulated by the activation of ROR-response elements within the I- κ B promoter because of the nuclear transcription ROR α . MLT decreases ROR α activity via an indirect relationship between the MT1 receptor and the RAR-related orphan receptor- α (ROR) [34]. The transcriptional activation of ROR α by calmodulin-dependent kinases (CaM) increases the transcription of I- κ B; thus, as MLT directly binds calmodulin, it prevents CaM activity allowing MLT to exercise its effects on antioxidative enzyme activity via receptor and non-receptor-mediated NF- κ B-related pathways [34].

Alongside preventing the destruction of the genome, there is substantial evidence to suggest that MLT may also aid in repairing mutations [35]. The implicated mechanisms of DNA damage-responsive pathways make use of base-excision repair (BER) [35,36]. However, as MLT does not directly alter the activity of three key glycosylases involved in BER, it is capable of reducing the repair time by 50% - one possible mechanism to achieve this is via acting on other BER-associated enzymes or their cofactors [26].

Damage-prevention and repair-enhancement properties present in MLT have been associated with limiting tumor cell initiation and

cancer frequency. The antioxidative properties of MLT are crucial for reduction in oxidative stress, which increases with cancer progression as the result of radiation treatment - this greatly increases the quality of life in cancer patients and enhances tumor control by allowing the use of a higher dose of radiation coupled with MLT therapy [37].

Anti-estrogenic properties and LA uptake reduction

Mammary breast cancers are linked to the levels of estrogen available either in its free circulating form or in the estrogen produced and accumulated by breast tissue [38]. The mechanisms for this link are argued to involve the various types of estrogens and metabolites acting as mutagens; in addition, the stimulatory effects of estrogen on cellular proliferation via ER (estrogen receptors alpha) drive the proliferation of mutations [39].

MLT orchestrates the seasonal reproduction period, and puberty depends on pineal gland function [40]. The lateness of appearance of secondary sexual characteristics is linked to high concentrations of MLT, whereas low concentrations may prevent puberty. These dysfunctions are commonly associated with the pineal gland damage and tumors [41]. The hypothalamus-pituitary-pineal gland neuroendocrine pathway illustrates that the concentration of the luteinizing hormone (LH), the follicle-stimulating hormone (FSH), testosterone, gonadotropin, and prolactin are linked to photoperiodicity [42-44]. Additionally, LH, FSH, and gonadotropin-releasing hormone synthesis cycles have 24-hour periods. MLT exercises direct effects on the levels of estrogen by acting on the ovaries via binding sites in human granulosa-luteal cells to modulate steroidogenesis in situ [45].

The anti-estrogenic properties of MLT depend on its ability to decrease the expression of ER and to inhibit the binding of the E2-ER complex to the estrogen response element (ERE) on DNA. These effects are exerted through MLT binding specifically to MT1 receptors [46,47]. In MCF-7 human breast cancer cells, the activation of MT1 receptors (but not MT2) enhances the response to the anti-estrogenic effects of MLT. MT1 receptors are located in the caveolae of MCF-7 cells [48]. Binding MLT to both MT1/MT2 activates various members of the Gi protein family, resulting in MAPK pathway modulation with effects on MEKs/ERKs and cAMP production downregulation. This causes modification of other downstream nuclear/cytosolic factors and drives cell differentiation, making cells less susceptible to malignancy, and reducing cell proliferation [49]. Both oncostatic and anti-proliferative effects are achieved via initiating signalling cascades downstream of MLT- MT1/MT2 receptor complexes.

A further mechanism whereby MLT decreases the growth of hormone-dependent cancers include its ability to inhibit aromatase, an enzyme which metabolizes androgen precursors to estrogens, as well as its action at the level of the ER, specifically ER [50]. This action reduces the stimulatory effects of endogenous estrogens on mammary cancer cell proliferation. Agents that inhibit aromatase are referred to as selective estrogen enzyme modulators (SEEM), while the latter are identified as selective estrogen receptor modulators (SERM). Thus, MLT-estrogen interactions involve direct effects and indirect effects when acting as SERM and SEEM. These anti-estrogenic effects of MLT limit the cell proliferation of hormone-dependent mammary cancer [39].

Inhibition of linoleic acid (LA) uptake by MLT is regarded as an antiproliferative mechanism. In addition to estrogen interactions, MLT acts to reduce the uptake of LA, an important initiating factor for the cellular cascades implicated in hormone-dependent breast cancers [51]. LA is a major essential fatty acid and is taken up into cells by specific fatty acid transporters. Interaction between MLT with MT1 and MT2 leads to the inhibition of adenylyl cyclase and reduction in the levels of cyclic AMP (cAMP), limiting the uptake of LA [51]. When circulating MLT levels are low, LA readily enters breast cancer cells where it is oxidized to 13-hydroxyoctadecadienoic acid (13-HODE), a mitogenic signaling molecule, by 15-lipoxygenase (15-LOX-1). It leads to the activation of the MEK-ERK1/2 pathway that promotes cell proliferation and tumor growth. When blood MLT levels are high, the indole acts via the membrane receptors to close the fatty acid transporter, limit LA uptake and shut down cell proliferation [51].

Immune system activation

The mechanisms in which MLT enhances the immune system is not fully understood, however there is ample evidence suggesting that the immune system plays at least three roles in tumor prevention: prevention of the formation of virus-induced tumors by eliminating viral infections, removal of pathogens to prevent inflammation, and identification and destruction of tumor cells that express tumor-specific antigens [52]. The existence of specific receptors for MLT in lymphoid cells confirms this effect in regulating and reinforcing the immune system response [8].

Alongside the pineal gland, MLT is synthesized in lymphoid organs such as the bone marrow, lymphocytes, and thymus, which are involved in the regulation of both innate and adaptive immune responses [53-55]. Innate immunity is the nonspecific first line of defense from microbes and transformed cells. It consists of macrophages, mast cells, dendritic cells (DCs), natural killer (NK) cells, etc. In contrast, adaptive immunity is antigen specific and is involved in the prevention and elimination of pathogens. There are two classes of adaptive immunity: cellular and humoral immunity, where cellular immunity uses T-cells that recognize cells displaying aberrant major histocompatibility complexes (MHCs), and humoral immunity uses B-cells that recognize pathogens or antigens found in the blood or lymph. The use of MLT has been shown to stimulate both innate and adaptive immunity [56].

MLT influences the immune system via MLT receptors, where both membrane and nuclear receptors have been found on leukocytes. Membrane and nuclear receptors allow MLT to induce cytokine production, modulate lymphocytes, restore impaired activity of T-helper (Th) cells in immuno depressed cells, promote T-lymphocyte proliferation, inhibit apoptosis of precursor B-cells in the bone marrow, and protect CD4+ T-cells from apoptosis. Furthermore, MLT prevents apoptosis of T-cell precursors in the thymus and it acts on T-cells throughout their development [56-60].

Experiments that inhibit MLT synthesis or secretion, including functional or surgical pinealectomy, show changes in the immune system, where production of cytokines is reduced [61]. Additionally, both *in vitro* and *in vivo* treatment with MLT has confirmed an enhanced immunological effect. Lymphocytes synthesize and secrete a substantial amount of MLT, further demonstrating the involvement

of MLT in the regulation of the immune system by acting as an intracrine, autocrine and paracrine molecule [55].

MLT is involved in the proliferation and maturation stages of monocytes, granulocytes and NK cells. This is observed with the enhanced production of progenitor cells of macrophages and granulocytes [62] and with the activation of monocytes leading to the secretion of IL-1-inducing cytotoxicity against tumor cells [63]. NK cells have the ability to recognize transformed cells and destroy them by inducing apoptosis and release interferon (IFN)- that leads to maturation of DCs, the antigen presenting cells, to promote the production of the cytotoxic T-cell CD8+ anti-tumor response [56]. MLT enhances the lytic function of NK cells, however, it is not fully understood whether the effect is via direct interaction of MLT and receptors on the surface of NK cells, or indirectly via increased IL-2 levels stimulated by MLT to enhance NK cell function [64-66].

MLT's ability to stimulate the immune system is linked to its capacity to enhance cytokine production together with antioxidant and anti-apoptotic effects. For instance, when peripheral blood mononuclear cells are cultured, MLT administration increased IL-2, IL-6 and IFN- production [67]. Moreover, long-term administration of MLT can increase the number of NK cells by enhancing the production of cytokines IL-2, IL-6, IL-12 and IFN- by T-helper cells containing MLT receptors [68-71]. In addition, MLT may enhance the immune response by increasing phagocytosis and antigen presentation [62], as following MLT administration, the antigen presentation of splenic macrophages to T-cells is enhanced and it is accompanied with an increase in MHC class II molecule expression and IL-1 and TNF- production [72]. MLT is thought to regulate immune function by acting on the immune-opioid network, by affecting the G protein-cAMP signal pathway and by regulating intracellular glutathione levels [73].

MLT stimulates the production of NK cells, monocytes and leukocytes, as well as increasing the production of IL-2, IL-6, IL-10, IL-12 and IFN- γ by the mononucleate cells, promoting a Th-1 lymphocyte response [8]. As MLT binds to membrane and nuclear receptors in Th cells, as well as stimulating the monocytes resulting in the production of the aforementioned cytokines, the immune response is upregulated [73]. Consequently, MLT is considered as an immune-enhancing agent.

Inhibition of tumor advancement: metastasis inhibition, anti-proliferative and pro-apoptotic activity

Metastasis is the process that involves the movement of neoplastic cells from the point of initial tumor formation to other tissues, organs or anatomical sites, and it is the primary cause of death in cancer patients. *In vitro* doses of MLT at 1 nM has been shown to decrease the invasiveness of 17-beta-estradiol (E2) induced MCF-7 human breast cancer cells, and it is correlated with an increase in expression of the cell-surface adhesion molecules E-cadherin and 1-integrin [74]. The anti-metastatic effect of MLT has been reproduced using three different clones of MCF-7 cells, which were treated with 0.1 or 1 nM of MLT. The anti-metastatic response was enhanced when MT1 was overexpressed, but reduced when MT1/MT2 receptor antagonist luzindole was added, suggesting that MLT exerts its effect on metastasis via specific membrane receptors [75]. Moreover, cancer patients with brain metastases, when treated with 20 mg of MLT per

day, together with supportive care, have shown an increased survival rate at 1 year compared to patients treated with supportive care alone [76].

Along with its anti-metastatic effects, MLT demonstrates anti-proliferative and pro-apoptotic activities in cancer cells as opposed to normal cells. The mechanisms of MLT in inhibiting cell proliferation vary across cancer cell types, including modulation of cell cycle duration: it promotes cell cycle arrest and enhances the prolongation of cell cycle duration in tumor cells, including in MCF-7 human breast cancer cells, human lymphoid malignancy cell lines (Burkitt's lymphoma, follicular B-cell lymphoma, and diffuse large B-cell lymphoma), the HepG2 hepatocarcinoma cell line, human osteosarcoma cell line MG-63, and HL-60 human myeloid cells, by expanding the G1 phase and delaying the entrance into S phase [36,77,78].

Expansion of the G1 phase reduces cell proliferation and allows entry of cells into G0, giving cells an opportunity to differentiate. Differentiated tumor cells are less aggressive and carry a better prognosis as expansion of the G1 phase allows the repair of DNA damage. Moreover, MLT administration has reduced the cytokinesis level of S-91 melanoma cells both *in vitro* and *in vivo* [79]. This anti-proliferative effect is cytostatic rather than a cytotoxic action as it is involved in modulating the cell cycle length. It is suggested that the inhibitory effect of MLT in the human osteosarcoma cell line MG-63 is due to a reduction in levels of cyclin B1, cyclin D1, cyclin-dependent kinase (CDK)1, and CDK4, each of which is important in cell cycle regulation [36]. MLT administration to human HepG2 hepatocarcinoma cells induced increased levels of p53 and its downstream effector p21, which is a potent inhibitor of cell cycle kinases causing the cell cycle arrest [80].

Evidence for MLT's direct receptor-mediated inhibitory effect on proliferation of cancer cells has been demonstrated when physiological and pharmaceutical doses of MLT inhibit the proliferation of human Choriocarcinoma JAr and JEG-3 cell lines: malignant tumor cells that arise from trophoblastic cells in the uterus [81,82]. The inhibition is correlated with the expression of the MT2 receptor but not the MT1 receptor. In contrast, the MT1 receptor plays a significant role in mediating the anti-proliferative effect of MLT in breast cancer [48]. Although G1/S transition delay has been observed in JAr cells, it is not the case in JEG-3 cells, suggesting the anti-proliferative effects of MLT are direct. Additionally, *in vivo* administration of MLT increased inhibition of JAr and JEG-3 xenograft tumors in nude mice and improved the survival of mice with choriocarcinoma, confirming the results of *in vitro* experiments [81].

MLT administration leads to cell cycle arrest in G2/M and increases the number of cells in G2/M of the cell cycle related to apoptosis. Further, MLT has been shown to inhibit apoptosis in normal cells, whereas it promotes apoptosis in tumor cells. In tumor cells, apoptosis occurs in high concentrations of MLT [77]. Experimental evidence has shown pro-apoptotic action of MLT on different cancer cell types both *in vitro* and *in vivo*, especially the pro-apoptotic effect of MLT on hematological cancers; apoptotic cell death was observed in Burkitt's lymphoma [82], acute myeloid leukemia [83], and acute lymphoid leukemia (RHE) cell lines [84]. Moreover, after treatment with MLT, the induction of apoptosis was

present in HepG2 liver cancer cells [80], Ewing's sarcoma cells [84] and rat pancreatic cancer cell lines [85]. MLT-induced apoptosis occurs via two pathways: the extrinsic and the intrinsic pathways that can act alone or in combination. The former leads to activation of caspase 8 and the latter leads to activation of caspase 9, both of which are involved in the activation of executioner caspases (3, 6 and 7) that further orchestrate the apoptotic pathways [86].

Anti-angiogenesis

Angiogenesis is the process of new blood vessel formation, and it is crucial for processes such as embryonic development, wound healing, and carcinogenesis. In the absence of vascular support, tumors may become necrotic or even apoptotic [87]. Rapidly proliferating cancer cells require an extensive network of blood vessels to ensure the constant supply of oxygen and nutrients. As the tumor mass increases, cancer cells become oxygen-deprived, thereby triggering hypoxia responses [87]. Such responses involve cascades activated by vascular endothelial growth factors (VEGFs) and the stromal-cell derived factor 1, resulting in growth and migration stimulation of endothelial cells, as well as recruitment of specific pro-angiogenic cells from the bone marrow [12]. As the VEGF family is present in both cancerous tissue and the adjacent stroma, they play an important role in neovascularization; tumor cells feed on the new blood vessels by producing VEGF and then secreting it into the surrounding tissue. Thus, processes of angiogenesis and neovascularization present logical targets for anticancer strategies [12,87].

Neovascularization, including tumor angiogenesis, is a four-step process: the tissue basement membrane is injured locally and there is immediate destruction and hypoxia, there is a migration of endothelial cells activated by angiogenic factors, endothelial cells proliferate and stabilize, and angiogenic factors continue to influence the angiogenic process [87]. Mutations in signalling pathways can promote tumor angiogenesis, for example, the PI3K/AKT (phosphatidylinositol-3-kinase) signalling pathway is important for regulating vasculature and angiogenesis processes. PI3K activation regulates the VEGF-A expression level in different types of cancer cells through HIF-1 (hypoxia-induced factor-1), ERK1/2, and NF- κ B activation to induce tumor angiogenesis [88]. The genetic alterations in the PI3K signalling molecules activate the PI3K signalling pathway involved in many types of human cancers, including thyroid, ovarian, colon, and breast cancers [88].

As MLT contains anti-angiogenic properties in various cell lines of cancer, including MDA-MB231 and PANC-1, endogenous VEGF suppression can be achieved and effectively maintained [12,89]. Both lines have provided positive results indicating the effectiveness of MLT as an anti-angiogenic treatment both *in vitro* and *in vivo*. Similarly, nude mice were grafted with foreign MDA-MB231 cell lines and demonstrated reduced tumor growth with decreased micro-capillary density in the tumor mass following daily MLT injected peritoneally [12]. Human studies of anti-angiogenic properties of MLT revealed positive results for both progressive-advanced and stable-advanced cancers, with stable-metastatic cancer patients showing a robust response correlated with the decline of serum-circulating VEGF [90]. Yet, MLT has the opposite effect of angiogenesis promotion due to increasing monocyte, cytokine and fibroblast proliferation rates in healing tissues, as observed in Wistar-albino rats [91]. This

antagonistic property of MLT in healthy cells makes its use in cancer therapy particularly attractive as only tumor cells will be the target of anti-angiogenic activity.

Epigenetic actions

Epigenetic changes are highly affected by the environment, and when in association with internal harmful effects, can induce nitro-oxidative stress in cells which can lead to several metabolic disorders, aging, and cancer [6,92]. Excess levels of folate activate methylation enzymes, which has been hypothesized to be crucial in the development of colon cancer [93]. The epigenotype is more flexible than the genotype; therefore, it is believed that epigenetic changes can play a substantial role in many human diseases. The development and progression of cancer implicates both epigenetic and genetic changes leading to the alteration of gene expression and cell phenotype [94].

The two molecular mechanisms involved in epigenetic regulation of gene expression are histone modification and DNA methylation. These mechanisms require several enzymes to assist in the process, including DNA methyltransferases (DNMTs), histone acetyltransferase (HATs) and histone deacetylases (HDACs) [95]. Multiple studies have provided sufficient evidence that increased activity of HATs and HDACs is associated with aberrant gene expression, which in turn leads to breast cancer [96]. The genes involved in metastasis (TIMPs) and limitless replicative potential (such as Cyclin D, p16, BRCA1, etc.) are methylated in breast cancer cells, whilst in non-carcinogenic cells they remain unmethylated [97].

Sirtuins are critical in cancer development as they play a dual role. For example, SIRT1 has been shown to activate stress defense and DNA repair mechanisms, thus allowing the preservation of the genomic integrity, yet overexpression can enhance tumor growth and promote cell survival in response to stress and drug resistance [94]. Strong inhibitors of sirtuins, such as salermidine, are pro-apoptotic due to the reactivation of suppressed genes [94], and recent findings suggest that increased levels of sirtuin lead to an increased number of different age-related tumors, which correlates with a reduced level of MLT production [98].

Epigenetic regulations can be controlled by MLT's ability to turn genes on or off. For example, MLT can regulate transcription factors modulated by nitro-oxidative and inflammatory conditions. Pathways controlled by NF- κ B and the activator protein-1 (AP-1) family directly activates pro-inflammatory cytokines and mediators like TNF-, interleukins, cyclooxygenase-2 (COX-2), cytokine-inducible nitric oxide synthase (iNOS), and matrix metalloproteinases (MMPs), which leads to inflammation [99]. These active transcription factors induce epigenetic processes by changing chromatin structure, either by acetylation of histones or methylation of DNA. Several studies have shown that MLT selectively inhibits iNOS and COX-2 [100] and MMPs [101], which a result of the suppression of NF- κ B is binding [102]. The suppression of the NF- κ B gene happens via recruitment of HDAC to its promoter region with MLT's action [103]. MLT inhibits p52 acetylation and binds to DNA, and therefore suppresses the expression of macrophage COX-2 and iNOS synthase [104].

Nuclear receptors (NR) are another class of protein that can inhibit or activate gene expression and regulate transcription, including estrogen receptors, retinoid acid receptors, peroxisome

proliferators-activated receptors (PPARs), retinoid X receptor (RXR), and glucocorticoid (GR). Enzymes that directly interact with NRs are co-regulators and can be either co-repressors or co-activators [105]. mRNA transcripts of nuclear MLT receptors (NMR) have been detected via using in situ hybridization of neuronal tissue and the pineal gland, which suggests that there is a genomic relationship between RZR/ROR receptors and MLT. These receptors are highly expressed in almost all normal tissues and are present in tumor cells such as breast, prostate and colon cancer. MLT modulates the transcriptional activity of ERa, GR and RAR receptors [105]. Similarly, when MCF-7 cells are treated with 1 mM and 100 mM of MLT, MLT downregulates EGR3 and POU4F2/Brn-3b genes via methylation, which are known tumor enhancers, and upregulates through methylation of GPC3 gene promoters, a known tumor suppressor [106], thus demonstrating that MLT can epigenetically affect cancer cells by playing a role in decreasing the development of tumors.

Conclusion

Evidence has been presented which suggests that MLT modulates estrogen and androgen activity, scavenges free radicals, inhibits cancer cell growth and proliferation, acts as an immunomodulator, and inhibits angiogenesis, whilst protecting from the precursors of hematopoiesis. Through its SERM and SEEM modulation, MLT inhibits the growth of androgen-sensitive prostate cancer cells, and, in certain cancer cell types, inhibits the uptake of linoleic acid, which prevents the formation of its mitogenic metabolite and inhibits the formation of endothelin-1. Additionally, MLT has been demonstrated to have radioprotective effects and scavenges free radicals in part through its stimulation of glutathione production.

Further evidence suggests that MLT may exert direct apoptotic effects by blocking cell cycle progression from the G phase to the S phase and by increasing gene expression in p53 and p21. Regarding the immune system, MLT increases the immunosurveillance system through stimulation of activity in lymphocytes, monocytes/macrophages, and natural killer cells. Lymphoid cells have also been shown to synthesize MLT, which regulates the immune system and it has been shown to increase the production of a number of cytokines. With the exception of free radical scavenging, these activities are thought to be mediated through MT1 and MT2 receptors. The nuclear binding sites for MLT have been identified in the majority of tissue types, and it is thought that MLT can further affect genomic activity at these sites.

MLT has been demonstrated to be an effective non-toxic molecule in both animal and human studies. MLT's versatility as an oncostatic agent is the result of its involvement in at least 6 distinct mechanisms at cellular and organismal levels. These effects are outlined in detail in Figure 1. All these properties of MLT suggest conducting further clinical trials of MLT and the use of MLT on cancer treatment.

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