

Melatonin as a Chronobiotic/Anticancer Agent: Cellular, Biochemical, and Molecular Mechanisms of Action and their Implications for Circadian-Based Cancer Therapy

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Abstract: Melatonin, as a new member of an expanding group of regulatory factors that control cell proliferation and loss, is the only known chronobiotic, hormonal regulator of neoplastic cell growth. At physiological circulating concentrations, this indoleamine is cytostatic and inhibits cancer cell proliferation *in vitro* via specific cell cycle effects. At pharmacological concentrations, melatonin exhibits cytotoxic activity in cancer cells. At both physiological and pharmacological concentrations, melatonin acts as a differentiating agent in some cancer cells and lowers their invasive and metastatic status through alterations in adhesion molecules and maintenance of gap junctional intercellular communication. In other cancer cell types, melatonin, either alone or in combination with other agents, induces apoptotic cell death. Biochemical and molecular mechanisms of melatonin's oncostatic action may include regulation of estrogen receptor expression and transactivation, calcium/calmodulin activity, protein kinase C activity, cytoskeletal architecture and function, intracellular redox status, melatonin receptor-mediated signal transduction cascades, and fatty acid transport and metabolism. A major mechanism mediating melatonin's circadian stage-dependent tumor growth inhibitory action is the suppression of epidermal growth factor receptor (EGFR)/mitogen-activated protein kinase (MAPK) activity. This occurs via melatonin receptor-mediated blockade of tumor linoleic acid uptake and its conversion to 13-hydroxyoctadecadienoic acid (13-HODE) which normally activates EGFR/MAPK mitogenic signaling. This represents a potentially unifying model for the chronobiological inhibitory regulation of cancer growth by melatonin in the maintenance of the host/cancer balance. It also provides the first biological explanation of melatonin-induced enhancement of the efficacy and reduced toxicity of chemo- and radiotherapy in cancer patients.

INTRODUCTION

Both normal and cancer cells are remarkably similar in that generally they contain the full array of molecules required for their proliferation, differentiation, survival and performance of specific functions. In the tissues of healthy individuals, there exists a delicate balance between cell proliferation and cell loss via apoptosis or terminal differentiation. The maintenance of normal cell physiology and metabolism requires that cells have the ability to detect, integrate and appropriately respond to a wide variety of external and internal signals that fluctuate within a multifrequency time structure [1]. A complex network of signal transduction and other cell regulatory pathways controlling proliferation and/or differentiation are temporally coordinated to precisely regulate the responses of normal cells to the environment. As envoys of the external and internal environment, stimulatory and inhibitory growth factors and hormones provide temporally-coordinated inputs to normal cells that establish the proper balance between cell proliferation and loss in the maintenance of the differentiated

state. On the other hand, the characteristic autonomous behavior and unchecked proliferation of cancer cells, which are derived from their normal counterparts, results from the loss of these regulatory influences and a breakdown in this "balancing act". Cancer cells are transformed by viruses, carcinogens and/or endogenous mutations rendering them less able to respond to their environment. Such malignant transformation is passed on to the progeny of cancer cells resulting in clonal expansion of the malignant phenotype and accumulation of cellular defects in growth control mechanisms. This results in further susceptibility to additional mutations leading to even greater loss of growth control, increasing the potential for metastasis and proliferation in otherwise hostile environments [2].

Phylogenetically, melatonin is an extremely old molecule produced in a wide variety of species from unicellular to vertebrate organisms. It is also a pleiotropic compound with a large repertoire of actions that operate in a diverse number of biological contexts. As the premier circadian hormone of darkness, its nocturnal production serves as a fundamental biological signal for apprising all the cells of the organism about the status of the environmental photoperiod and biological time [3,4]. As a newly acknowledged player on the ever expanding team of regulatory factors that control cell proliferation and loss,

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melatonin holds the unique position of being the only known chronobiotic regulator of neoplastic cell growth [5,6]. Although this review will briefly survey the evidence for melatonin as an antineoplastic molecule, it will primarily focus on the mechanisms by which this indoleamine is currently thought to control cancer cell proliferation and tumor growth in the context of biological timing. Consideration will be given to these mechanistic strategies in the maintenance of the host/cancer balance and their implications for melatonin supplementation in the chronoprevention and -therapy of cancer.

CELLULAR MECHANISMS OF ACTION

Melatonin and Cancer Cell Proliferation

Results from most *in vitro* studies support melatonin's role as an antiproliferative or oncostatic molecule in a variety of human and some murine cancer cell lines (i.e., breast, endometrial, ovarian, choriocarcinoma, prostate, colon, melanoma, neuroblastoma). These antiproliferative effects are primarily exerted over a concentration range that encompasses levels of melatonin found in the blood circulation during the night [5-7]. The question of what constitutes a true physiological concentration of melatonin requires serious re-evaluation. There is significant uptake and retention of melatonin by tissues and cells resulting in local concentrations of the indole that are orders of magnitude higher than the peak blood concentrations produced by the pineal gland at night [8].

Generally speaking, cells plated at low densities and exhibiting slow growth rates elicit little or no inhibitory response to doses of melatonin ranging from the subphysiologic to pharmacologic [9-11]. In fact, some cancer cell lines such as PC3 androgen-independent human prostate cancer cells even exhibit a stimulatory growth response to physiological and pharmacological doses of melatonin depending on the cell density [12]. At higher plating densities and growth rates, most cancer cell lines exhibit an inhibitory response to melatonin at a variety of doses particularly in the physiological range (100 pM to 1 nM) [13]. The dose-response of tumor cells to inhibitory concentrations of melatonin varies from a bell-shaped to a linear pattern depending on the cell line tested as well as the culture conditions used. For example, human breast cancer (MCF-7), human neuroblastoma, human uveal melanoma and murine colon carcinoma cells exhibit the bell-shaped pattern with the inhibitory response basically restricted to the physiological range and no or little response at subphysiological or pharmacological concentrations. Other cell lines exhibit a linear dose-response such that the degree of growth suppression increases with the dose of melatonin. Even in the same cell line (i.e., MCF-7) the dose-response can change from a bell-shape, when cells are cultured in monolayer on plastic, to a linear pattern, when they are grown under anchorage-independent conditions [9-13].

Although a few laboratories report no effect of physiological melatonin on cancer cell proliferation *in vitro*, some of these same investigators have observed cytostatic effects at pharmacological doses. This has sparked some

degree of controversy regarding melatonin's designation as an oncostatic hormone *in vitro* [7]. However, different stocks of cancer cell lines sharing the same progenitor but propagated under different growth conditions in different laboratories may select for the growth of specific subclones that are more or less responsive to the oncostatic activity of melatonin and other biologically active molecules [14]. Culture conditions that may select for the growth of specific clones include, but are not limited to the type of media, batch of serum and plastic substratum used. There is also a significant variation in the incubation conditions used from one melatonin/cancer study to another including the use of different plating densities, serum content, duration of incubation, frequency of media changes and type of culture system.

In addition to the cell type used, different culture conditions can result in a quite complex pattern of cell proliferative responses to different concentrations of melatonin resulting in dose-response curves that are either bell-shaped, biphasic or linear as alluded to above. Even the mode of cellular exposure to melatonin during incubation has a marked impact on the degree of growth inhibition. For example, a time-varying, pulsatile exposure of breast cancer cells to physiological levels of melatonin that mimicks its circadian pattern *in vivo* results in significantly greater oncostatic potency than a flat exposure to one physiological concentration of the indole [9,10].

Therefore, considering the substantial lack of standardization or duplication of exact culture conditions among different studies, it is not surprising that a handful of negative reports have occurred in the literature with respect to the reproducibility of the melatonin growth inhibitory response [7]. In spite of these discrepancies, it can be generally stated that under most *in vitro* conditions in a variety of cancer cell types, physiological levels of melatonin decrease the rate of cell proliferation while higher concentrations appear to be either cytostatic or cytotoxic. The role of the physiological nocturnal melatonin surge as an natural oncostatic signal is supported by *in vivo* experiments in which tumor development and growth is accelerated in animals which have been either pinealectomized or exposed to light during darkness [5-7].

Melatonin and the Path to Differentiation

In some human cancer cell lines and transplantable murine tumors, melatonin's antiproliferative effect results from an inhibition of cell cycle kinetics [7,9,15,16]. The cell cycle consists of different stages including the initiation and completion of DNA replication (S) phase, the phase of mitosis (M) and the gap phases (G_0/G_1 and G_2) between S and M phases. G_0 is a resting state characteristic of the normal, nondividing differentiated cells. When cells leave this quiescent state to subsequently undergo multiple cycles of cell division, they initially enter G_1 in which protein and RNA synthesis is active. This is a very complex phase of the cell cycle in which cells must traverse a critical checkpoint called the restriction (R) point at which cells become irreversibly committed to another round of DNA replication. Promotion of cells through the R point requires

the activation of proteins called G₁ cyclins which include the D type cyclins in mid G₁, and cyclin E in late G₁ [2]. In MCF-7 human breast cancer cells [7,9-11] and JAr human choriocarcinoma cells [16], physiological melatonin levels delay the progression of cells from the G₁ to the S phase of the cell cycle. In the case of MCF-7 cells, this melatonin-induced transition delay significantly prolongs the duration of the cell cycle by 15%. As the majority of cells pile up in G₀/G₁ and the proportion of cells in S phase decreases, melatonin also suppresses DNA synthesis in the remaining cells that have progressed to the S phase [9-11].

Tumor growth ultimately reflects a disruption in the delicate balance between cell proliferation and apoptotic cell loss and/or permanent cell cycle arrest with the balance markedly tipping toward proliferation. A central regulator of apoptosis and cell cycle arrest is the tumor suppressor gene *p53* which codes for a tetrameric protein that binds to specific sequences thereby transactivating a group of genes involved in apoptosis and/or cell cycle arrest (e.g., *p21/Waf1*, *bax*). *p53* turns on the transcription of *p21/Waf1* which binds to and inhibits the ability of cyclin D1 and E-dependent kinases to hypophosphorylate and inactivate retinoblastoma (*Rb*) tumor protein resulting in the blockade of cell progression from G₁ to S [2,17]. Since melatonin up-regulates the expression of *p53* and *p21/Waf1* proteins in MCF-7 cells *in vitro* [7,9-11], this may be an important mechanism by which melatonin causes a cell cycle transition delay at the G₁ - S interface in this cell line as well as in JAr human choriocarcinoma cells [16]. The accumulation of cells in G₁ provides a greater opportunity for them to enter the quiescent, non-dividing state (G₀) which is typical of terminally-differentiated cells [2]. This would suggest that melatonin's antiproliferative effects on tumor cells may be due in part to its ability to shift the balance from proliferation to differentiation. Supportive evidence for this hypothesis comes from studies showing that pharmacological levels of melatonin *in vivo*, although having no antitumor effect, facilitate the differentiation of pluripotent stem cells in mouse teratocarcinoma along neural and muscle cell lineages [18]. Subsequent investigations have demonstrated that physiological blood concentrations of melatonin promote the differentiation of MCF-7 cells in culture, as assessed by morphological and morphometric techniques which would be consistent with melatonin's ability to stifle the growth progression of these cells from G₀/G₁ to S-phase [7,9-11].

Experimental findings related to melatonin's influence on the metastatic behavior of MCF-7 cells also support the differentiation hypothesis. For example, physiological melatonin reduces the invasiveness of MCF-7 cells by decreasing their capacity for attachment to a basement membrane and by blocking estradiol (E₂)-induced cell adhesion. Additionally, melatonin reduces the chemotactic response of MCF-7 cells toward the extracellular matrix (ECM) protein fibronectin and attenuates E₂-induced cell migration [9-11]. Tumor cell motility and invasion are adhesion-dependent phenomena that rely on the presence of cell surface adhesion molecules for both cell-matrix and cell-to-cell interactions [2]. Therefore, it is not surprising that melatonin increases the MCF-7 cell expression of E-cadherin and β_1 -integrin since these molecules are important for cell-

to-cell contact and cell-matrix interactions, respectively. The expression of these ECM molecules has been inversely correlated with tumor cell invasiveness and differentiation *in vitro* and melatonin apparently shifts MCF-7 cells to a lower invasive status by promoting the differentiation of these cells [9-11].

Another aspect and hallmark of the differentiated state is the ability of normal cells to establish and maintain proper intercellular communication via maintenance of the structural and functional integrity of intercellular gap junctional contacts. Such junctional contacts ensure that critical regulatory information is passed between and among terminally-differentiated, non-proliferating cells. Local gap junctional intercellular communication is fostered by cell adhesion molecules such as E-cadherin which facilitates cell-to-cell interactions including connexon-to-connexon interactions. On the other hand, a progressive loss of E-cadherin expression correlates closely with a progressive loss of gap junctional intercellular communication. Cancer cells experience a breakdown in intercellular communication because of defective cell adhesion and/or malfunctioning gap junctional contacts [2,19]. Growth factors or chemical agents that promote tumor growth progression down-regulate gap junctional function while some anti-tumor promoting agents up-regulate and maintain intercellular gap junctional communication; melatonin falls into this latter category. For example, physiological to pharmacological concentrations of melatonin significantly increase gap junctional contacts among mouse embryo fibroblasts (C3H/10T1/2), MCF-7 cells and rat hepatocytes in culture [20-22]. At pharmacological concentrations melatonin markedly induces the expression of Cx32 gap junction protein in rat hepatocytes [22]. It has been postulated that melatonin acts as a differentiating agent to suppress cancer cell proliferation by increasing cell-to-cell interactions via its ability to: 1) augment the expression of E-cadherin and Cx32 gap junctional protein, and 2) stabilize membrane fluidity and thus re-establishing gap junctional integrity [21,22].

Probably the most compelling evidence for melatonin as a differentiating agent comes from studies showing that physiological nighttime blood concentrations of melatonin induce the cytodifferentiation of human neuroblastoma cells (SK-N-SH) in culture as manifested by smaller cell and nuclear size and increased neurite outgrowth. Quiescent, terminally-differentiated cells in the G₀ resting state are known to be smaller than undifferentiated, proliferating cells progressing through the cell cycle [11]. Equally provocative is the demonstration that pharmacological melatonin concentrations accelerate the differentiation and mineralization of rat pre-osteoblastic cells (MC3T3-E1). It also rapidly induces the expression of bone marker protein genes such as bone sialoprotein (BSP), alkaline phosphatase (ALP), osteopontin (OPN) and osteocalcin (OC), via transcriptional activation, in MC3T3 pre-osteoblasts as well as in fully differentiated rat osteoblast-like osteosarcoma cells (17/2.8) [23]. Interestingly, the differentiating effect of melatonin on these cells appears to involve membrane bound melatonin receptors (see below) since a melatonin receptor antagonist prevents melatonin-induced differentiation. In non-neuronal Chinese hamster ovary (CHO) cells, stably-transfected with melatonin receptors, pharmacological

melatonin causes cytoskeletal changes that instigate the development of long, filamentous "neurite-like" processes by these cells whereas no such effect occurs in non-transfected CHO cells [24]. These results are reminiscent of the differentiating effects of melatonin on neurite outgrowth in neuroblastoma cells cited above [11].

The above discussion has focused on the differentiating action of melatonin as a mechanism for its growth inhibition of already initiated cancer cells. However, it is important to acknowledge melatonin's role in helping to promote the differentiated state of developing normal cells that are susceptible to cancer-initiating insults posed by environmental factors particularly chemical carcinogens. Currently, the only model system in which this has been studied is the mammary gland ductal system of murine species. In developing rodent mammary glands, terminal end buds (TEBs), like their counterparts (i.e., terminal ductal lobular units) in human mammary glands of nulliparous women, are the most undifferentiated structures of the developing ductal system and are highly susceptible to tumor initiation by chemical carcinogens particularly when these structures are present at a high density. The susceptibility of the mammary gland to neoplastic transformation is due to the high proliferative rate of the epithelium which has a high DNA-labelling and mitotic index. Treatment of rats or mice with pharmacological doses of melatonin decreases DNA synthesis in and the number of TEBs resulting in a more differentiated ductal system that is more resistant to cancer initiation by exposure to carcinogens. In transgenic mice carrying the *N-ras* protooncogene under transcriptional control of mouse mammary tumor virus long terminal repeat (MMTV-LTR), the mammary glands are predisposed to developing cancers. The mammary glands of these mice possess abundant premalignant hyperplastic alveolar nodules (HANs) with dysplastic epithelial cells that overexpress *N-ras* protein. Treatment of these mice with pharmacological amounts of melatonin decrease the density of HANs, eliminate dysplastic epithelial cells and reduce the expression of *N-ras* providing further evidence for melatonin's differentiating action. Whether melatonin helps to maintain a differentiated state in other cells susceptible to neoplastic transformation is unknown [9,10].

Of course, another mechanism by which melatonin might inhibit carcinogen-initiated mammary cancer is via its ability to stymie the accumulation of DNA adducts formed by carcinogens that cause damage to and permanent alterations in DNA which lead to neoplastic transformation. This may be accomplished directly via melatonin's ability to act as a potent free radical scavenger and/or via its indirect antioxidant actions to detoxify carcinogens via activation of the glutathione and related metabolic pathways [25]. Since DNA can repair itself in response to some carcinogenic insults through mechanisms such as DNA-dependent protein kinase (DNA-PK) [2], it is conceivable that melatonin not only protects DNA from damage in the first place but also promotes its repair via DNA-PK once damage has occurred. This would serve to limit the amount of permanently lesioned DNA and restrict or totally prevent cancer initiation.

Melatonin and the Path to Apoptosis

Physiological melatonin-induced increases in *p53* expression, which can trigger apoptosis in some cell types, suggest that, in addition to inducing cell cycle arrest, this indoleamine may induce apoptosis in cancer cells [9-11]. In fact, melatonin has been reported to induce apoptotic cell death in MCF-7 cells, as indicated by an increase in DNA strand breaks, by one laboratory [9-11] but not another [26]. Moreover, no changes in the expression of either *bax*, (apoptosis activator gene), *bcl-2* or *bcl-x_L* (apoptosis suppressor gene) genes are detected in these cells in response to melatonin alone. However, in MCF-7 cells treated sequentially with retinoic acid and physiological melatonin, not only is there clear evidence of programmed cell death but the expression of *bax* is augmented while the expression of *bcl-2* is diminished [26].

In vivo, pharmacological doses of melatonin suppress clonal proliferation of stem cells during both the early and late stages of chemically-induced colon carcinogenesis in rats. Interestingly, melatonin exerts a biphasic effect on apoptosis during colon carcinogenesis by suppressing it during the early stages while promoting it during the later stages. Pathobiological studies of specimens of primary human gastrointestinal and lung tumors also show that there is a positive correlation between melatonin immunoreactive and apoptotic tumor cells [27]. In a transplantable murine colon tumor (Colon 38) model system, the administration of daily afternoon injections of pharmacological doses of melatonin substantially increases the apoptotic index of these tumors following six days of treatment [28]. Thus, under certain circumstances and in certain tumor types, melatonin-induced apoptotic cell death may play a mechanistic role in cancer cell growth inhibition.

BIOCHEMICAL AND MOLECULAR MECHANISMS OF ACTION

Melatonin Regulation of the Estrogen Response Pathway in Human Breast Cancer Cells

Most of what is currently known about the cellular and molecular mechanisms by which melatonin inhibits tumor cell growth is based on extensive *in vitro* studies in estrogen receptor (ER) positive, also referred to as ER α , MCF-7 human breast cancer cells. This subject has been extensively reviewed in more detail in several review articles to which the reader is referred [6,7,9-11,26,29]. Melatonin has no effect on the proliferation of ER- human breast cancer cells indicating that the estrogen-response pathway is a critical component of the pathway mediating melatonin's oncostatic effect in breast cancer cells. Physiological melatonin not only inhibits the mitogenic effects of estradiol (E₂) on MCF-7 cells, but it down-regulates the expression of the ER by suppressing the transcription of the ER gene resulting in a time-dependent reduction in ER mRNA and protein levels. Melatonin itself does not bind directly to the ER indicating that its ability to regulate ER expression is exerted via indirect mechanisms rather than competing with E₂ for the hormone-binding domain of the ER [26,29].

Interestingly, a potentially important relationship exists between melatonin levels and the ER content of breast cancer tissue. For example, the amplitude of the circulating nocturnal melatonin peak is substantially blunted in women with ER+ breast cancer as compared with either age-matched healthy women or women with ER- disease [30]. Furthermore, the magnitude of the nocturnal melatonin peak is inversely correlated with the ER content of breast cancer tissue whereas the melatonin content of breast cancer tissue is positively correlated with the ER content [31]. These clinical findings lend additional credence to a connection between the physiological melatonin signal and the estrogen response pathway in human breast cancer.

The ER is a member of a superfamily of ligand-inducible transcription factors which bind to specific recognition sequences [estrogen responsive elements (EREs)] in the DNA of responsive genes which then become transcriptionally activated to produce mRNAs and proteins involved in cell proliferation, differentiation and physiology. Two distinct regions or domains of the receptor [i.e., activation factor-1 (AF-1) and AF-2] regulate transcription both independently and synergistically depending on the promoter and cell type. In the absence of E₂, the inactive receptor is complexed with a variety of proteins that block its ability to interact with EREs whereas in the presence of E₂, the receptor experiences a conformational change that allows it to bind to coactivators and initiate the transcription of target genes. Estradiol-induced phosphorylation of amino acid residues located in the amino terminal A/B and ligand binding domains of the ER also increases ER binding to the

ERE and transcriptional activation [26,32,33]. Physiological melatonin blocks the ability of E₂ to stimulate the binding of the ER to the ERE of DNA in nuclear extracts of MCF-7 cells. Although physiological melatonin decreases the basal phosphorylation state of the ER by activating phosphatases or inhibiting phosphorylases, it apparently does not destabilize the E₂-ER-ERE complex through inhibition of E₂-induced phosphorylation [26,35].

Physiological melatonin cannot itself affect the transcriptional activity of the ER in the absence of E₂. However, when melatonin pre-treatment is followed by either epidermal growth factor (EGF) or insulin-like growth factor-1 (IGF-1) co-treatment in MCF-7 cells, ligand independent transactivation of the ER occurs very rapidly in association with an increase in mitogen activated protein kinase (MAPK). It has been postulated that ER transactivation by melatonin plus EGF renders the receptor less sensitive to E₂ and thus less efficient in regulating the transcripton of E₂-responsive genes that are critical for MCF-7 cell proliferation [26].

Another member of the steroid/thyroid hormone receptor superfamily of nuclear transcription factors that is related to retinoic acid receptors is retinoic orphan receptor (ROR α). ROR α isoforms are expressed in MCF-7 cells but do not bind melatonin. However, physiological melatonin suppresses the transactivation and DNA-binding activity of ROR α while having no effect on ROR α protein levels in MCF-7 cells; this may occur via melatonin modulation of the Ca²⁺/CaM signaling pathway [26,36] (see below).

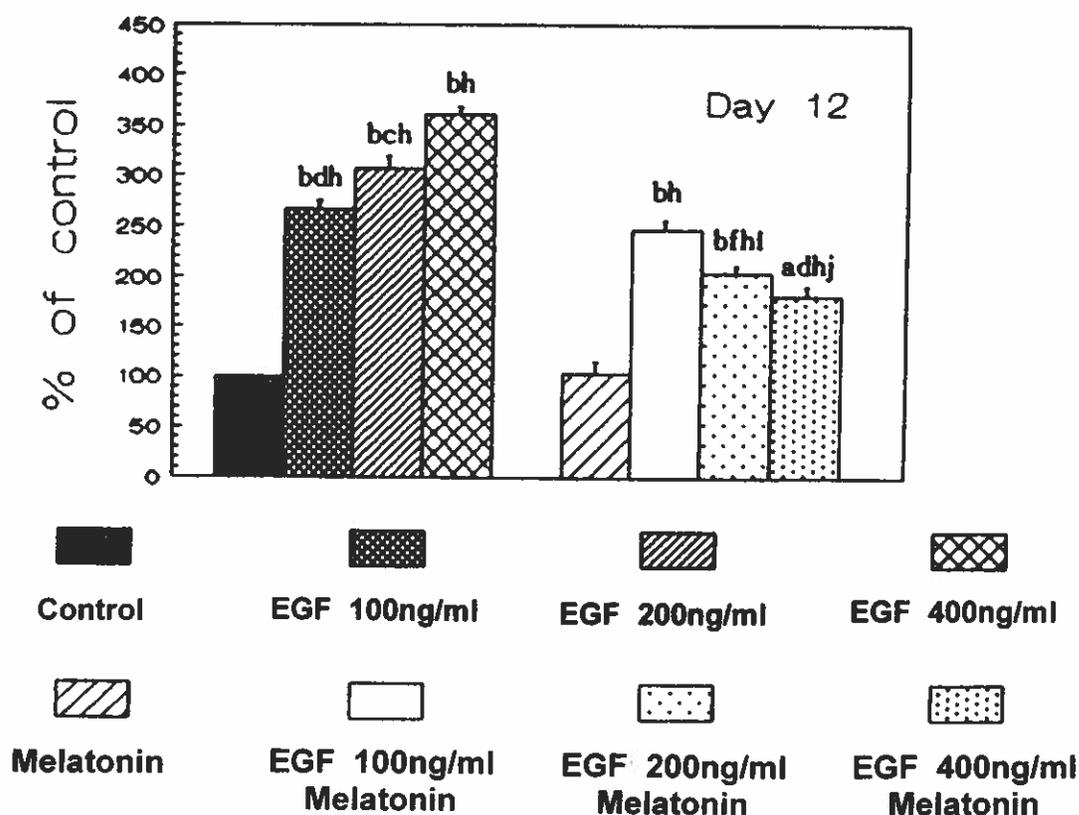


Fig. (1). Effects of physiological melatonin (1 nM) on epidermal growth factor (EGF)-stimulated MCF-7 cells growth over 12 days in serum-free, chemically-defined medium.

The E₂-induced proliferation of breast cancer cells, particularly MCF-7 cells, may be mediated by E₂ induction of endocrine, autocrine and paracrine growth factors such as transforming growth factor α (TGF α) and prolactin (PRL) as well as by E₂ inhibition of growth inhibitory factors such as TGF β . Physiological melatonin levels inhibit the expression of TGF α while stimulating the expression of TGF β by MCF-7 cells in culture [26]. Also in MCF-7 cells, physiological melatonin blocks or attenuates the mitogenic effects of both PRL and EGF "Fig. (1)" which is structurally and functionally homologous with TGF α [5-7]. Thus, melatonin's ability to suppress the estrogen response pathway in breast cancer cells may be mediated via the modulation of the synthesis of E₂-induced growth factors as well as by altering their capacity to act on their cellular targets.

Melatonin and the Potential Role of Calcium/Calmodulin (Ca²⁺/CaM) and Protein Kinase C and Effects on Cytoskeletal Structure and Function

As the major Ca²⁺ receptor in eukaryotic cells, CaM plays an important role in not only mediating many Ca²⁺-dependent events but in the proliferation of normal and neoplastic cells via intracellular processes including cell cycle progression and cytoskeletal integrity [36,37]. For example, Ca²⁺-activated CaM is involved in the initiation of the S-phase of the cell cycle, initiation and completion of the M-phase, cell cycle-related gene expression, and cytokinesis. Calmodulin concentrations affect progression through the G₁/G₀-phase and mitosis and thus affects the rate of cell cycle progression. Calmodulin is also necessary for the reentry of quiescent cells from G₀ into the cell cycle and for traversing the G₁/S and G₂/M boundaries. Progression through the cell cycle involves the enlargement and depolymerization of microtubules and agents which elevate CaM levels stimulate microtubular enlargement and depolymerization and thus cell proliferation whereas CaM antagonists have the opposite effects [36].

The antiproliferative effects of physiological melatonin in certain neoplastic cell lines (i.e., murine neuroblastoma NIE-115) may be due to increased degradation of CaM due to melatonin binding to CaM as well as to melatonin-induced changes in the intracellular distribution of CaM from the cytosol to the cytoskeletal membrane fraction [38]. Ca²⁺/CaM promotes microtubule depolymerization and thus DNA synthesis and cell proliferation via phosphorylation of tubulin and microtubule-associated proteins (MAPs) through CaM-dependent protein kinase [36,37]. Melatonin, acting as a CaM antagonist, may inhibit cytoskeletal disruption by binding to CaM and blocking MAPs/CaM and tubulin/CaM complex formation. Therefore, melatonin, by suppressing CaM levels, inducing changes in the subcellular distribution of CaM and inhibiting CaM-dependent multiprotein kinase II activity and autophosphorylation, may block reentry of cells into the cell cycle and mitosis [38].

Protein kinase C (PKC) is the major intracellular receptor for diacylglycerol (DAG), the second messenger involved in phospholipid metabolism and signaling. Several PKC isoenzymes are Ca²⁺-dependent and the long-term regulation

of PKC isoenzymes appears to be important in the regulation of cancer cell proliferation since prolonged activation of PKC via phorbol esters promotes tumor growth. Cytoskeletal proteins such as MAPs are phosphorylated by PKC thus inducing microtubular changes required for mitotic spindle and aster formation. In NIE-115 neuroblastoma cells, prolonged exposure to physiological melatonin inhibits cell proliferation while short-term melatonin exposure activates PKC inducing a translocation of Ca²⁺-dependent PKC α from the cytosol to the membrane cytoskeletal fraction. However, prolonged exposure to physiological melatonin down-regulates PKC which may contribute to its oncostatic action in these cells [38].

Intermediate filaments such as vimentin are intimately involved in the cell adhesion, migration and invasiveness of metastatic cells. Physiological melatonin concentrations cause changes in vimentin intermediate filament structure and increase the phosphorylation of vimentin which may contribute to a reduced metastatic potential of cancer cells [38].

As mentioned above, Ca²⁺ exerts a potent mitogenic effect on cancer cells *in vitro* including MCF-7 cells [39]. Peptide growth factors as well as estrogen itself increase intracellular Ca²⁺ levels ostensibly via stimulating the influx of Ca²⁺ into cells through non-genomic mechanisms involving cell surface receptors [36]. Interestingly, CaM itself appears to be an ER-binding protein and directly modulates L-type voltage sensitive Ca²⁺ channels. Melatonin, in addition to its multiple effects on CaM, acts as blocker of voltage-sensitive Ca²⁺ channels perhaps via its effects on CaM levels and/or function [6]. With respect to cancer cell growth, physiological melatonin inhibits Ca²⁺-stimulated MCF-7 cell proliferation when CaCl₂ is added to Ca²⁺-deficient medium. In fact, melatonin loses its oncostatic effect in Ca²⁺-deficient medium whereas the addition of Ca²⁺ back to the culture medium not only restores melatonin's oncostatic action but actually enhances it [6]. In androgen-responsive LNCaP human prostate cancer cells, pharmacological and near physiological concentrations of melatonin inhibit cell proliferation and attenuate Ca²⁺ influx induced by either 5 α -dihydrotestosterone (DHT) or E₂ [40]. These results indicate that melatonin's oncostatic action may be mediated, in part, in certain cancer cell types by its ability to alter Ca²⁺/CaM metabolism, block Ca²⁺ influx and thus suppress microtubule polymerization.

Melatonin and the Potential Role of Cellular Redox Mechanisms

Reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide and the hydroxyl radical, generated as a result of normal cellular metabolism, are thought to participate in a variety of cellular/molecular signaling mechanisms that regulate transcriptional/translational processes, cell growth, differentiation and apoptosis [41,42]. In cancer cells, ROS, organic peroxides and radicals can act as tumor growth promoters. Increased cellular concentrations of these molecular species can create a prooxidant state that can lead to cancer initiation or to the growth promotion of already initiated cells [43]. Growth factors have been shown

to increase the intracellular concentrations of ROS which then stimulate mitogenesis via the induction of the phosphorylation of receptor protein tyrosine kinases (RTKs) such as the EGF receptor (EGFR) as well as MAPK also referred to as extracellular-regulated kinase (ERK) [41,42].

While ROS and free radicals can act as tumor promoters, antioxidant and free radical scavengers can have anti-tumor promoting and anticarcinogenic activity [43]. Compelling evidence now exists for melatonin's role as an indirect antioxidant and potent, direct free radical scavenger at both pharmacological and physiological concentration. It's role as an inhibitor of neoplastic transformation and cancer initiation via its ability to reduce DNA damage by scavenging free radicals has been acknowledged above [25,44]. Another mechanism by which pharmacological melatonin may inhibit cancer growth promotion may be through its ability to directly (i.e., via free radical scavenging) or indirectly (i.e., activation of antioxidative enzymes) neutralize ROS such hydrogen peroxide [45] that act to promote the growth of cancer cells. This may be one explanation for melatonin's ability to suppress MCF-7 cell growth by EGF since growth factors may stimulate the RTK-MAPK growth response pathway via the generation of ROS [41,42]. Paradoxically, melatonin, at both physiological and pharmacological concentrations, activates the production of ROS by human monocytes to levels that are cytotoxic to cancer cells with which they are co-incubated [45]. These findings indicate that melatonin can promote free

radical generation if the cellular context is appropriate and it is in the interests of host survival.

The existing redox state of the cancer cell, as determined by the integrity of the glutathione (GSH) metabolic pathway, may play a major role in determining whether melatonin exerts its physiological antiproliferative effect at least in the case of MCF-7 human breast cancer cells. As a potent and abundant intracellular antioxidant, GSH provides the intracellular compartment with a reducing environment. Physiological melatonin not only increases the intracellular levels of GSH in association with an inhibition of MCF-7 cells growth "Fig. (2)", but the indole's oncostatic action is totally incapacitated in these cells when GSH synthesis is pharmacologically blocked by L-buthionine sulfoximine (L-BSO). This compound inhibits γ -glutamylcysteine synthetase, the enzyme that is rate-limiting for GSH synthesis. Interestingly, ER- human breast cancer cells, which do not ordinarily respond to melatonin's antiproliferative effects, are rendered responsive to melatonin when GSH-S-transferase, another important enzyme for GSH metabolism, is inactivated by ethacrynic acid [46]. However, the integrity of redox state of the cancer cell as governed by GSH is apparently not important for the cytotoxic effects of pharmacological concentrations of melatonin in other cancer cells such as ME-180 human cervical cancer cells [47].

In MCF-7 cells, cytotoxicity induced by physiological and pharmacological concentrations of melatonin may involve

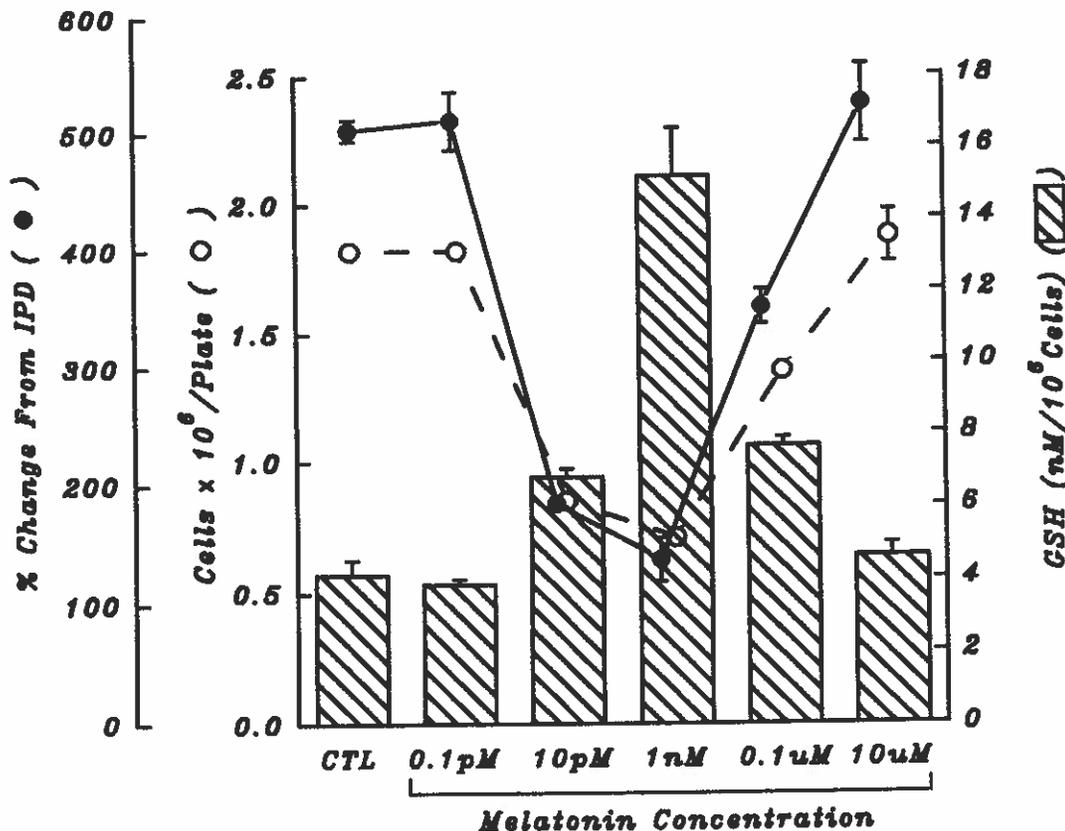


Fig. (2). Dose-response effects of various melatonin concentrations on MCF-7 cell growth [% change from the initial plating density (IPD)] and total glutathione (i.e., oxidized and reduced) (GSH) concentrations following five days of incubation in DMEM containing 10% fetal bovine serum.

uncoupling of oxidative phosphorylation as evidenced by an acceleration in mitochondrial electron transport chain activity as reflected by increased mitochondrial oxygen consumption and succinate dehydrogenase activity (complex II) in the face of decreasing cytochrome *c* oxidase (complex IV) activity and intracellular ATP levels [48]. *In vivo*, melatonin's inhibition of dimethylbenzanthracene (DMBA)-induced mammary carcinogenesis in rats may be due to its ability to elevate GSH levels in mammary and liver tissue in an effort to detoxify DMBA. Melatonin may also detoxify DMBA by suppressing phase I enzymes cytochromes b5 and P450 [49].

Another avenue by which melatonin may exert its antiproliferative effects on MCF-7 breast cancer cells through cellular redox mechanisms could involve nitric oxide (NO). Nitric oxide has emerged as an extremely important intercellular and intracellular signaling molecule in a variety of physiological contexts and can act as either a free radical or antioxidant species [50,51]. Nitric oxide has cytostatic antiproliferative effects in cancer cells ostensibly via an inhibition of ribonucleotide reductase, the rate-limiting step in DNA synthesis. It has also been implicated in a number of mechanisms of cellular toxicity including apoptosis [6,7]. The redox state of the intracellular milieu can favor certain redox forms of NO which may target specific cellular processes such as DNA synthesis resulting in a cytostatic response in cancer cells [51]. Since melatonin can alter the redox status of MCF-7 breast cancer cells via alterations in GSH levels [46], "Fig. (2)", we examined whether NO might be involved in melatonin's oncostatic mechanism of action. Nitric oxide is synthesized from L-arginine by NO synthase (NOS) the activity of which is suppressed by melatonin [52]. Since NO released from human monocytes are cytotoxic to cancer cells in culture [45], we determined whether the inhibition of the endogenous production of NO by MCF-7 cells, with the NOS inhibitor N-monomethyl-L-arginine (NMMA), would affect physiological melatonin's oncostatic action in this cell line. While NMMA completely blocks melatonin's antiproliferative effect, sodium nitroprusside (SNP) a NO donor, abrogates NMMA's effect on melatonin-induced oncostasis in a dose-dependent manner [6,7]. These findings indicate that melatonin may stimulate NO production or potentiate NO's cytostatic action in MCF-7 cells. Support for both postulates derive from studies showing the ability of physiological melatonin to stimulate NO production by human monocytes [45] and the ability of NO to directly interact with and nitrosate melatonin to produce N-nitrosomelatonin [53]. It is possible that this nitrosated melatonin conjugate may be an even more potent antiproliferative molecule than either NO or melatonin alone.

Melatonin and Melatonin Receptor-Mediated Signal Transduction Involvement in Cancer Cell Growth and Differentiation

Cellular networks that normally control cell proliferation and/or differentiation consist primarily of signal transduction and cell regulatory pathways that tightly regulate cellular responses to the environment. Cells, including cancer cells, have the ability to sense a myriad of extracellular signals primarily via plasma membrane receptors that transmit

information to the intracellular compartment resulting in either a stimulation or inhibition of cell proliferation and/or differentiation [2]. Cell surface-associated G protein-coupled receptors (GPCRs) play a major role in mediating mitogenic signaling in normal and cancer cells through the modulation of MAPK activity. Mitogen-activated protein kinases, specifically the extracellular signal-regulated kinases ERK1 and ERK2 (MAPK), are activated via proximal kinases, Raf-1 and B-Raf [MAPkinase kinase kinase (MAPKKK)] that phosphorylate and in turn activate MEK1 and MEK2 [MAPkinase kinase (MAPKK)]. These MAPKs are involved in signal transduction events regulating cell proliferation and differentiation and may mediate pleiotropic responses in the membrane, cytoplasm, nucleus or cytoskeleton depending on the cellular context. Upon activation, MAPKs translocate to the nucleus where they phosphorylate and thereby activate nuclear transcription factors involved in DNA synthesis and mitogenesis [54].

Earlier work demonstrated that membranes from cancer cells, particularly murine melanoma cell lines, exhibited high affinity (i.e., picomolar) and low capacity specific binding of 2[¹²⁵I]iodomelatonin. However, membranes from other cancer cell types such as murine breast cancer tissue and androgen-independent PC3 human prostate cancer exhibited lower affinity binding 2[¹²⁵I]iodomelatonin binding (i.e., nanomolar) [12]. These findings strongly suggested that the direct oncostatic or cytotoxic effects of melatonin might occur via bona fide cell surface melatonin receptors. Of course, three high affinity melatonin receptor subtypes were eventually cloned and found to be new members of the superfamily of GPCRs. The receptor subtypes are designated as MT₁ (formerly Mel_{1a}) and MT₂ (formerly Mel_{1b}), which are found in mammalian tissues, and Mel_{1c} which is found in non-mammalian tissues [55]. Melatonin receptors are thought to be intimately involved in melatonin regulation of variety of biological functions including circadian rhythms, seasonal reproduction, immune activity, vascular reactivity and retinal physiology. A major mechanism by which melatonin may modulate many of these physiological functions is through activation of particular receptor subtypes resulting in the inhibition of intracellular cAMP accumulation via pertussis toxin (PTX)-sensitive inhibitory G proteins that suppress adenylyl cyclase activity [55,56]. Signal transduction responses to melatonin activation of specific melatonin receptor subtypes, coupled to a variety of other G proteins, include the downregulation of cGMP levels, PKC and MAPK activity, and upregulation of phosphoinositide hydrolysis, PKC activity, intracellular Ca²⁺ mobilization, potassium ion channels, arachidonic acid production and MAPK activity [56].

Since the cloning of the MT₁ and MT₂, a number of studies have demonstrated the presence of these melatonin receptor subtypes in cancer cells and tissues. The presence of mRNA transcripts and protein for any of the melatonin receptor subtypes in cancer cells was first demonstrated for MT₁ in MCF-7 human breast cancer cells via RT-PCR and western blot analysis, respectively. The presence of mRNA transcripts or protein for either MT₁ or MT₂ was not detected in ER- MDA-MB-231 human breast cancer cells which may help explain why physiological melatonin levels

fail to induce an antiproliferative effect in these and other ER- breast cancer cell lines [26]. Interestingly, different stocks of MCF-7 cells express different levels of both ER and MT₁ receptors; the level of MT₁ expression directly correlates with the level of ER expression. Estrogen receptor and MT₁ receptor expression in turn correlates with melatonin responsiveness such that cells expressing higher levels of both ER and MT₁ receptors are more sensitive to the oncostatic effects of melatonin [14]. Involvement of MT₁ receptors in physiological melatonin inhibition of the estrogen response pathway is further supported by the fact that PTX inhibits E₂-induced transactivation of the ER as well as melatonin + EGF or IGF-1-induced ER transactivation [26]. Most interesting is a recent report that a low magnetic flux density, 50 Hz electromagnetic field (MF) blocks physiological melatonin's capacity to inhibit cell proliferation and forskolin-induced cAMP accumulation in MF-sensitive MCF-7 cells [57]. This apparently occurs via MF-induced uncoupling of the MT₁ GPCR, its associated G_{i/o} protein and adenylyl cyclase from cAMP providing further evidence for the importance of this signal transduction cascade in mediating melatonin's oncostatic action in MCF-7 cells.

Subsequently, other cancer cell lines have been shown, by RT-PCR, to express mRNAs for either MT₁ or MT₂. Like MCF-7 cells, androgen-dependent LNCaP human prostate cancer cells and human prostate cancer tissues express transcripts and protein for MT₁ but not MT₂ [58]. High affinity melatonin agonists as well as physiological melatonin itself, inhibit LNCaP cell proliferation in some studies suggesting a MT₁-mediated response [40]. Additionally, pharmacological inhibition of PKC activity with bisindolylmaleimide (GF-109203) prolongs the ability of physiological melatonin to suppress LNCaP cell proliferation. This suggests a PKC-induced inactivation of the MT₁ receptor, the expression of which is reduced by phorbol ester-induced PKC activation via a cAMP-dependent mechanism [12]. However, in other investigations, while physiological melatonin inhibited cell growth, high affinity melatonin agonists were ineffective [58]. In one of these studies, melatonin failed to suppress forskolin-stimulated cAMP accumulation in LNCaP cells suggesting that melatonin's direct antiproliferative action in this cell line is not mediated via a melatonin GPCR. In contrast, JAr human choriocarcinoma cells [16] and human uveal melanoma cells [59] express mRNA transcripts for MT₂ but not MT₁. The pharmacological MT₂ receptor antagonist luzindole has been used to demonstrate the presence of MT₂ receptors in ER+ Ishikawa human endometrial adenocarcinoma cells by its ability to block the oncostatic action of physiological melatonin [60].

In androgen-independent PC3 human prostate cancer cells exhibiting relatively low affinity 2[¹²⁵I]iodomelatonin binding, physiological melatonin's effects on cell growth, cGMP and cAMP are either stimulatory or inhibitory depending upon the cell density. Regardless of the cell density conditions used, PTX blocks all of melatonin's effects on 2[¹²⁵I]iodomelatonin binding, cell growth and cyclic nucleotide levels indicating that these actions are mediated via melatonin GPCRs. The differential and cell density-dependent effects of melatonin on signal

transduction and cell growth indicates that melatonin may be a tumor growth modulator in some cancer cell lines depending on the cellular context in which it finds itself [12].

The MT₁ and MT₂ receptor subtypes have also been implicated in mediating melatonin's ability to promote cellular differentiation in normal and neoplastic cells. Both luzindole and PTX block the ability of pharmacological concentrations of melatonin to cause the differentiation of rat pre-osteoblastic cells (MC3T3-E1) as evidenced by the inhibition of melatonin-induced expression of several bone marker proteins (see above) [23]. These findings indicate an important role for the MT₂ receptor in mediating the differentiation of this cell type. In NIH3T3 mouse fibroblasts, stably-transfected with both MT₁ and MT₂ receptors, exposure to very high concentrations of melatonin suppress cell transformation (i.e., formation of foci in culture) and proliferation induced by the mere presence of both receptor subtypes in these cells [61].

In cancer cells such as the N1E-115 mouse neuroblastoma cell line, MT₁ receptors are endogenously expressed and mediate melatonin-induced differentiation as evidenced by increased neurite formation. This is blocked by simultaneous treatment with PTX and is not mimicked by a subtype-specific MT₂ activator [62]. Similar differentiating effects of pharmacological melatonin are observed in non-neuronal Chinese hamster ovary (CHO) cells stably-transfected with MT₁ receptors which develop neurite-like processes in response to melatonin. The differentiating effects of pharmacological melatonin in these cells may occur via MT₁ GPCR-induced phosphorylation and activation of MEK/MAPK [24]. Thus, melatonin's capacity to act as a differentiating agent on cancer cells may be dependent on the appropriate expression and function of MT₁ and/or MT₂ GPCR subtypes as well as on the cell type involved.

Melatonin and Melatonin Receptor-Mediated Signal Transduction in the Control of Tumor Polyunsaturated Fatty Acid Metabolism and Growth *In Vivo*

As is readily evident from the previous discussion, most of the cellular and molecular mechanisms of melatonin's action on cancer cell proliferation and differentiation have been elucidated in studies performed *in vitro* primarily on cancer cell lines of human and murine origin. As important as these *in vitro* approaches are in broadening our understanding of how melatonin works on cancer cells, they suffer from an inability to address the chronobiological features of melatonin's mechanisms of action in cancer growth regulation and therapy within the integrative and systemic context of the host/cancer balance. Such an approach requires an *in vivo* model system that allows the integration of circadian melatonin regulation with the study of melatonin's mechanisms of action, via biochemical and molecular signal transduction pathways at the level of the tumor *in situ*. Retaining the systemic context in melatonin cancer research is critical to obtaining a true understanding of melatonin's chronobiotic anticancer mechanisms that

optimize the host/cancer balance in favor of the host and to the detriment of the cancer.

Such a model system and experimental approach has been developed in recent years in our laboratory in Cooperstown, NY. This tumor model system takes advantage of the fact that either murine or human tumors can be grown in appropriate host animals in a tissue-isolated manner allowing for reliable, serial measurements of tumor growth coupled with pharmacological, biochemical and molecular manipulations and assays of signal transduction mechanisms [63-66]. With this strategy, all the systemic, integrative influences of nutritional and photoperiodic interactions with the circadian system, in general, and the melatonin rhythm-generating system, in particular, are preserved. In our system, a small piece of transplantable tumor tissue is attached to and grown on the end of a surgically-created vascular stalk consisting of the superficial epigastric artery and vein in the inguinal fossa of the host animal. The tumor is encased in an envelope of parafilm to prevent the tumor from becoming attached to adjacent tissues and invaded by other host vascular structures. Hence, this tissue-isolated tumor is allowed to grow with its arterial supply and venous drainage coming exclusively from the epigastric vessels. Not only can accurate tumor growth measurements be made on a regular basis during tumorigenesis, but at appropriate points along the tumor's growth curve, the vessels can be cannulated for arteriovenous difference measurements, across the tumor, of various biochemical factors and products important in tumor growth and metabolism. In addition, the tumor can be perfused *in situ* with physiological and/or pharmacological agents that alter specific signal transduction pathways. Assessments of the biochemical and molecular consequences of such manipulations can then be made via arteriovenous difference measurements across the tumor as well as through direct biochemical and molecular analyses of the tumor tissue itself.

In most of our studies, we have used male Buffalo rats bearing implants of tissue-isolated Morris rat hepatoma 7288CTC, which among a variety of other transplantable tumors, is characterized by a unique growth requirement for linoleic acid. Linoleic acid (C18:2n6), an n6 polyunsaturated fatty acid, is abundant in the Western diet and the major polyunsaturated fatty acid consumed in the human diet. It is the major fatty acid in safflower, sunflower, corn, soy bean, and cottonseed oils, accounting for greater than 50% of the total fatty acid content in these oils. Although it is an essential fatty acid, consumption of modest amounts, equivalent to 1% of total calories, is adequate to protect against essential fatty acid deficiency [67].

It has become increasingly apparent that linoleic acid derived from dietary sources and/or endogenous lipid stores serves not only as an energy source for tumor growth, but more importantly, as a specific tumor growth signaling molecule. Several investigations have shown that high fat diets containing linoleic acid as the major fatty acid increase the growth rates of transplantable murine tumors as well as of human breast cancer and prostate cancer xenografts in immunodeficient mice. Increased blood concentrations of linoleic acid following its dietary intake results in a

corresponding increase in the arterial supply to and uptake of this fatty acid by tissue-isolated hepatoma 7288CTC, culminating in a two-fold or more increase in the growth rate. Of the total linoleic acid taken up by hepatoma 7288CTC *in vivo* during tumor perfusion *in situ*, 1 - 10% is oxidized within the tumor to 13-hydroxyoctadecadienoic acid (13-HODE) by 15-lipoxygenase activity [67]. Treatment of tumor-bearing animals with the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) inhibits the formation of 13-HODE and causes tumor growth regression without altering the rate of tumor linoleic acid uptake. Perfusion of tumors *in situ* with NDGA-containing arterial blood does not affect tumor linoleic acid uptake but inhibits the formation of 13-HODE and decreases [³H]thymidine incorporation into DNA by the tumor. The addition of 13-HODE to arterial blood containing NDGA completely restores [³H]thymidine incorporation to pre-NDGA levels [68].

The activity of the lipoxygenase responsible for the conversion of linoleic acid to 13-HODE is regulated by the tyrosine kinase moiety of the EGFR such that binding of EGF to its cognate receptor stimulates lipoxygenase activity resulting in an increased rate of 13-HODE formation from linoleic acid. Following its formation, 13-HODE enhances the EGF-induced autophosphorylation of the EGFR as well as the tyrosine phosphorylation of key downstream signal transduction proteins including MEK/MAPK. It is through this mechanism that 13-HODE augments the mitogenic effects of EGF. Thus, linoleic acid serves as a substrate and 13-HODE is the mitogenic signaling molecule responsible for linoleic acid-dependent tumor growth [69].

Although normal and neoplastic cells take up fatty acids, there is a lack of a clear understanding of the mechanisms by which this occurs. It is commonly accepted that fatty acids enter cells by simple diffusion, but mounting evidence supports a role for specific fatty acid transporters as well. Currently, three fatty acid transporters have been identified and characterized including two that are integral membrane proteins: fatty acid translocase (FAT) and fatty acid transport protein (FATP). The plasma membrane-bound fatty acid binding protein (FABP) is a peripheral membrane protein similar to or identical to mitochondrial aspartate aminotransferase. Each of these proteins increases fatty acid uptake when expressed in various cell lines. FATP, which is a member of a family of 5 - 6 related isoforms represented among several tissues within a species and with homologues in different species, is highly expressed in tissues with high rates of fatty acid oxidation and metabolism such as heart, adipose tissue and skeletal muscle [67]. Additionally, several members of the FATP family exhibit very long chain acyl-CoA synthetase activity with the ability to esterify very long chain fatty acids upon their transport into the cell while simultaneously blocking their egress from the cell [70]. As compared with normal liver, FATP is overexpressed in tissue-isolated rat hepatoma 7288CTC and presumably is a major fatty acid transport protein in this tumor [71].

Initial experiments in our laboratory demonstrated that exposure of rats, bearing tissue-isolated tumors, to either constant bright light 24-hr/day or to dim light during the dark phase of a 12 hour light:12 hour dark cycle

(12L:12D) markedly stimulates the growth of these tumors presumably due to increased rates of tumor linoleic acid uptake and 13-HODE production. Since constant light or dim light during darkness completely suppresses the nocturnal melatonin peak, these results suggest that the endogenous melatonin signal may play an important role in restraining tumorigenesis in these animals under standard laboratory photoperiodic conditions [72]. Therefore, we determined whether there was a relationship between the endogenous melatonin rhythm and the uptake of linoleic acid and its metabolism to 13-HODE by hepatoma 7288CTC over a 24-hr period particularly since plasma fatty acids are elevated during the dark phase when food intake is highest. If, in fact, the physiological melatonin signal exerts an inhibitory control over tumor linoleic acid uptake and metabolism as the light exposure experiments strongly suggest, is there a circadian rhythm of suppression of tumor fatty acid metabolism that correlates with the endogenous melatonin circadian rhythm? And if so, is this tumor rhythm "driven" by the endogenous melatonin rhythm?

By measuring tumor metabolism every four hours during both the light and dark phases over a 24-hr period, we found that these tumors did indeed exhibit a prominent rhythm in linoleic acid uptake and its conversion to 13-HODE that is temporally correlated with the circadian melatonin rhythm. During the light phase, when circulating melatonin levels are lowest, tumor linoleic acid uptake and 13-HODE production are highest. Conversely, during the dark phase when plasma

melatonin is at its peak, tumor fatty acid uptake and metabolism is at its nadir. Furthermore, in pinealectomized animals without a circadian melatonin signal, tumor fatty acid uptake and 13-HODE production are persistently high for the entire 24-hr period indicating that the central, endogenous circadian melatonin signal is "driving" the peripheral circadian rhythm of tumor fatty acid metabolism. Subsequent experiments, in which hepatomas were perfused *in situ* with physiological, nocturnal circulating concentrations of melatonin, demonstrated that melatonin has a rapid, reversible, specific and direct inhibitory effect on tumor linoleic acid uptake and metabolism to 13-HODE [71]. Dose-response studies show that these effects are saturable at around 1 nM melatonin with no additional inhibition being achieved at 1 μ M (Blask *et al.*, unpublished results). These effects of melatonin on tumor fatty acid metabolism are not limited to hepatoma 7288CTC since similar results were obtained in tissue-isolated rat mammary adenocarcinoma and MCF-7 human breast cancer xenografts perfused *in situ* with melatonin. These findings provide unequivocal support for our conclusion that the endogenous melatonin rhythm is responsible for a reciprocal rhythm in tumor linoleic metabolism. They also confirm that tumor metabolism is not static throughout the day but oscillates in a tightly-controlled manner in response to the circadian melatonin signal from the pineal gland [71].

Not only does hepatoma 7288CTC overexpress mRNA transcripts for FATP but, as assessed by RT-PCR, this

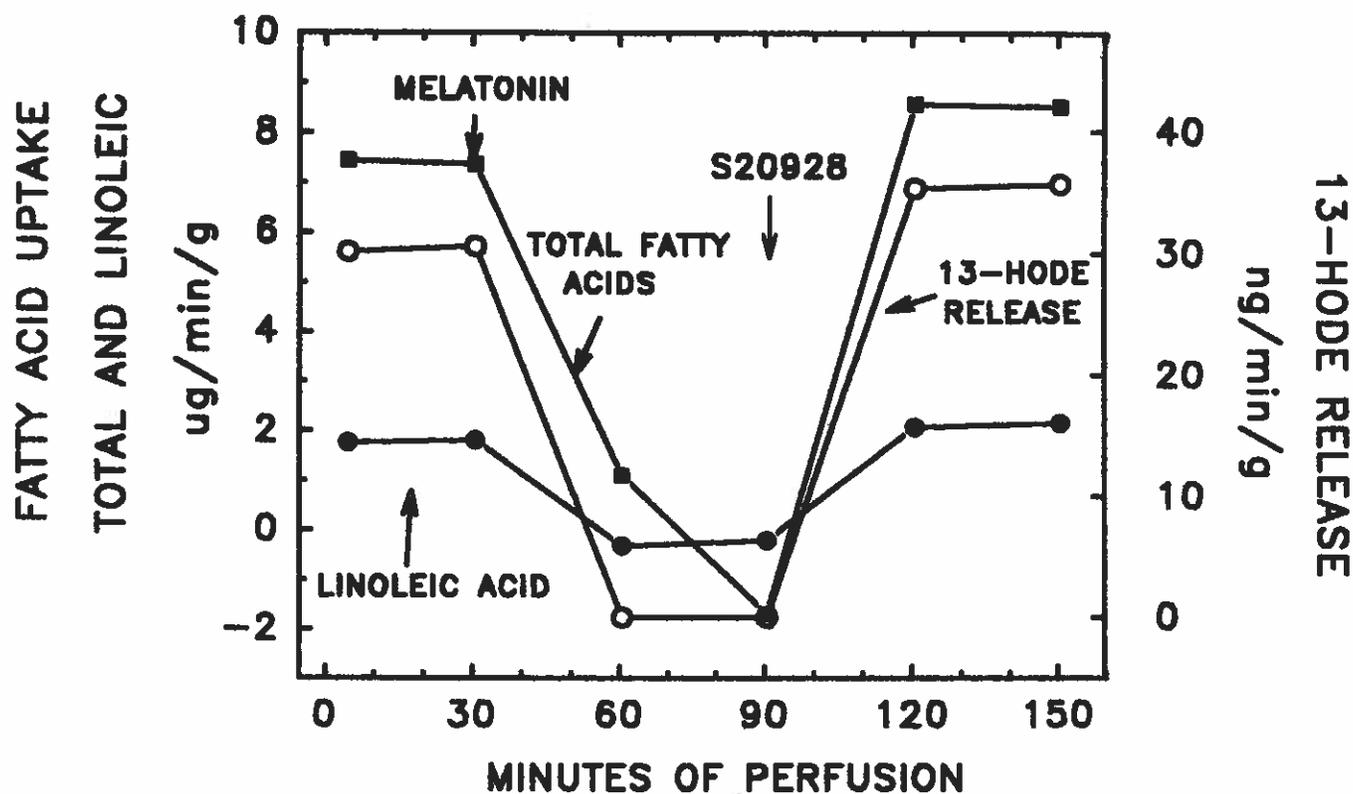


Fig. (3). Rates of total fatty acid and linoleic acid uptake and 13-hydroxyoctadecadienoic acid (13-HODE) release from tissue-isolated hepatoma 7288CTC perfused *in situ* with physiological melatonin (1 nM) followed by melatonin receptor antagonist S-20928 (1 nM).

tumor express mRNA transcripts for both MT₁ and MT₂ receptors (Blask *et al.*, unpublished results). This indicates that melatonin may be exerting its suppressive effects on tumor linoleic acid metabolism via melatonin GPCR inhibition of cAMP. Additional tumor perfusion experiments bear this out since the melatonin-induced inhibition of tumor linoleic acid uptake and 13-HODE formation is blocked by the melatonin receptor antagonist S20928 (Fig. (3)), PTX, forskolin and 8-Br-cAMP. Similar results were obtained in tissue-isolated mammary adenocarcinomas with these pharmacological agents (Blask *et al.*, unpublished) indicating that melatonin's inhibitory action on tumor fatty is operating through a signal transduction mechanism common to other tumor types. We have not yet determined whether one or both melatonin receptor subtypes mediate this response.

Stimulation of cAMP accumulation with either PTX or forskolin, or perfusing tumors with cAMP itself reverses or prevents the inhibitory effects of melatonin on tumor linoleic acid uptake and metabolism to 13-HODE. This indicates that cAMP is crucial for maintaining the functional integrity of the fatty acid transport mechanism mediated by FATP. Considering that elevated levels of cAMP have been documented in several types of malignancies including hepatoma, melanoma and mammary carcinoma, our results make a convincing argument that a facilitated fatty acid transport mechanism via FATP is operating in these tumors [71].

Similar to the effects of constant light exposure or dim light exposure during darkness, pinealectomy of rats maintained on a 12L:12D photoperiod 1) induces a two-fold increase in tumor growth rates (Fig. (4)), 2) markedly augments the rates of tumor linoleic acid uptake and 13-HODE formation, and 3) increases the total fatty acid

content of these tumors as compared with sham-operated controls. These findings provide further convincing evidence for an inhibitory role of the endogenous melatonin rhythm in tumor fatty acid metabolism and growth. Conversely, treatment of tumor-bearing rats with daily, late afternoon subcutaneous injections of pharmacological doses of melatonin (i.e., one to two hours prior to lights off) or administration of melatonin in the diet causes 1) a substantial suppression of tumor growth (Fig. (4)), 2) inhibition of tumor linoleic acid uptake and metabolism to 13-HODE, and 3) a reduction in tumor total fatty acid content. However, these results still do not absolutely prove whether melatonin inhibition of tumor growth is the result or the cause of suppressed fatty acid metabolism. Tumor perfusion *in situ* with physiological melatonin suppresses not only linoleic acid uptake and 13-HODE formation but tumor DNA content and [³H]thymidine incorporation into DNA as well. Additionally, the inhibitory effects of melatonin on cell proliferation are prevented by the melatonin receptor antagonist S20928, PTX, forskolin, 8-Br-cAMP and 13-HODE. These data provide unequivocal proof that melatonin-induced tumor growth inhibition results from a melatonin GPCR-mediated blockade of tumor linoleic acid uptake and its conversion to 13-HODE and not the other way around [71].

Chronobiology of Melatonin as an Anticancer Agent

In view of the fact that melatonin is a chronobiotic, it is not surprising that a number of studies show that its anticancer action *in vivo* is predicated on the time of day it is administered. Generally, melatonin administered to tumor-bearing animals in the late afternoon, a few hours prior to lights off, is quite effective in suppressing tumorigenesis while morning melatonin therapy, a few hours after lights

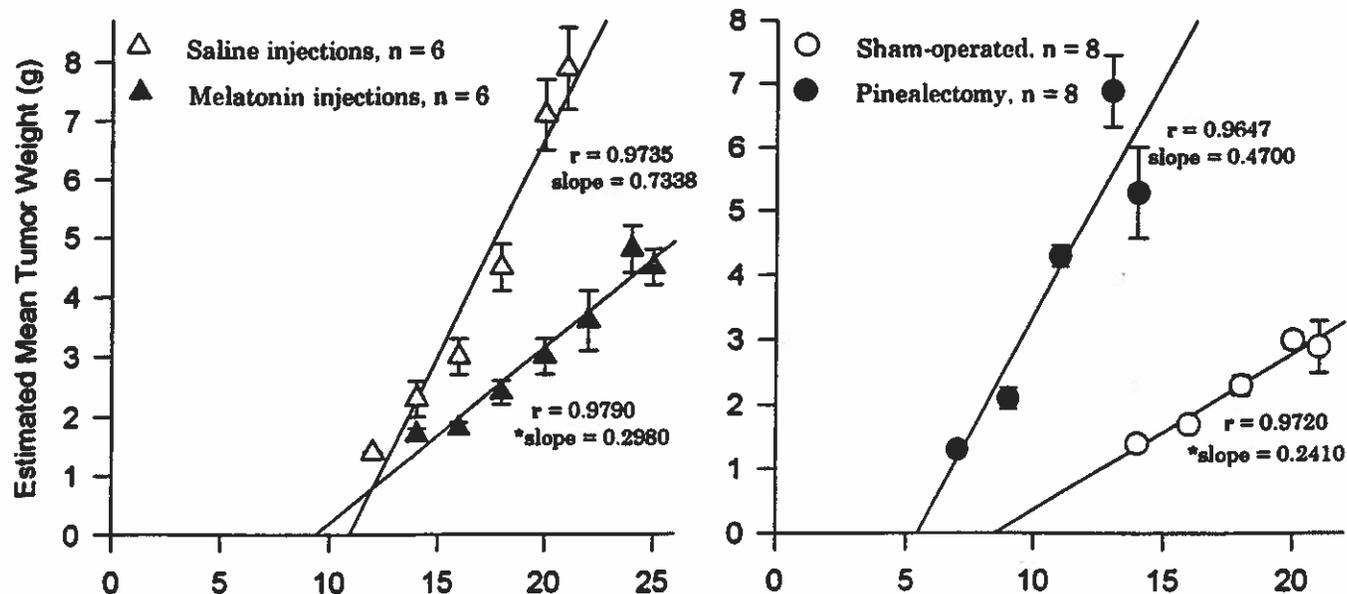


Fig. (4). Estimated mean tumor weights of tissue-isolated hepatoma 7288CTC in 1) rats treated with daily, late afternoon (two hour prior to lights off) s.c. melatonin (200 μ g/day) or vehicle injections (left panel) or in 2) rats that were either surgically pinealectomized or sham-operated (right panel).

on, is either stimulatory or has no effect on tumor growth [5]. In tissue-isolated hepatoma 7288CTC, unlike later afternoon melatonin therapy, morning injections of melatonin (i.e., two to three hours after lights on) fail to inhibit tumor growth or linoleic acid uptake and metabolism. This suggests that these tumors exhibit a circadian rhythm of sensitivity to the suppressive effects of exogenously administered melatonin on tumor growth and fatty acid metabolism [73].

Perhaps such a rhythm of tumor sensitivity is driven by the endogenous melatonin signal itself. Preliminary data showing that either morning or late afternoon injections of melatonin are equally effective in inhibiting tumor growth and linoleic acid uptake and metabolism in tumor-bearing animals exposed to constant light and lacking a melatonin rhythm (Blask *et al.*, unpublished results) supports such a conclusion. This phenomenon could potentially be explained on the basis of a circadian rhythm in the density of melatonin receptors similar to what occurs in peripheral tissues such as the spleen [74]. This rhythm appears to be driven by the endogenous melatonin signal since melatonin receptor number is lowest just after lights on. Ostensibly, this is due to melatonin receptor down-regulation by the previous night's melatonin peak. During the light phase, as circulating melatonin levels remain low, receptor numbers are gradually regenerated to their maximal density by the end of the light period; the cycle then repeats itself. However, under conditions of constant light or pinealectomy, melatonin receptor levels remain elevated throughout the 24-hr period.

We postulate that a melatonin-driven circadian rhythm in melatonin receptor number may also occur in tumor tissues such as hepatoma 7288CTC. This could help explain melatonin's ineffectiveness in the morning when tumor receptor levels, theoretically, are lowest whereas in the late afternoon, when receptor density is highest, melatonin would be most effective in inhibiting tumor fatty acid metabolism and growth. Under constant light conditions, melatonin is effective at either time of day since, hypothetically, tumor melatonin receptors would be constantly high due to the absence of the endogenous melatonin peak and receptor down-regulation. However, such a hypothesis of circadian tumor regulation by melatonin still awaits experimental verification.

Although quite speculative at this point in time, another potentially important aspect of linoleic acid and melatonin regulation of the chronobiology of tumor growth may involve circadian clock gene expression and function. For example, clock and clock-related genes are expressed in a circadian manner in a rat hepatoma cell line (H35) *in vitro* in response to a high concentrations of serum [75] which contain, among other things, linoleic acid, growth factors and melatonin. Furthermore, food and thus, nutrient restriction, synchronizes the circadian oscillation of clock and clock-related gene expression in liver, *in vivo*, independent of the central circadian pacemaker [i.e., the suprachiasmatic nucleus (SCN)] [76]. Moreover, in NIH-3T3 mouse fibroblasts, circadian oscillation of clock gene expression is triggered by activation of the MEK/MAPK signaling

cassette and inhibited by pharmacological deactivation of this pathway [77]. Therefore, it is conceivable that there may also be a melatonin-driven, circadian oscillation in tumor clock gene expression *in vivo* that is important for tumor metabolism and growth. This could be accomplished via melatonin's ability to restrict tumor linoleic acid uptake and 13-HODE formation resulting in an alternating day/night, activation/deactivation of MEK/MAPK signaling and, ostensibly, clock gene expression and function. Such a scenario, if true, would imply the temporal coordination and integration, both centrally (i.e., SCN) and peripherally (i.e., tumor tissue), of circadian metabolic and neuroendocrine signals that may regulate tumor circadian clock gene activity, metabolism and growth.

CONCLUSIONS

A Unifying, Mechanistic Model for Chronobiological Inhibitory Cancer Growth Regulation by Melatonin

Based on the evidence obtained primarily from our studies on hepatoma 7288CTC and human breast cancer xenografts, we believe that the anticancer effect of either endogenous or exogenous melatonin is dependent on the time of day it interacts with tumor cells. For example, the beginning of the light phase, when circulating melatonin levels and presumably numbers of unoccupied tumor melatonin receptors are lowest, maximal rates of tumor growth occur because high cAMP levels enhance FATP activity. This may occur via cAMP-dependent protein kinase A-mediated phosphorylation of FATP. Entry of fatty acids including linoleic acid into tumor cells is thus facilitated by an overexpressed and highly active FATP. Epidermal growth factor present in host arterial blood and tumor interstitial fluid binds to tumor EGFRs to stimulate the MEK/MAPK mitogenic signaling pathway and tyrosine kinase activity of the EGFR to increase 15-lipoxygenase activity. Thus, a portion of the linoleic acid that enters tumor cells is converted to the mitogenically active metabolite 13-HODE due to EGF/EGFR-activated lipoxygenase activity. The increasing levels of 13-HODE formed, resulting from unfettered cAMP-dependent FATP-mediated transport of linoleic acid, feeds back positively on the MEK/MAPK pathway to further augment EGF-induced mitogenesis and provoke a vicious cycle of cancer cell proliferation. Like a "runaway train", mitogenesis continues, even as unencumbered tumor melatonin receptor numbers theoretically increase to peak at the end of the light phase because of still low melatonin levels.

Shortly after dark onset, circulating melatonin levels begin to rise causing up-regulated melatonin receptors to become increasingly occupied. As melatonin increases to peak in the middle of the dark phase, melatonin receptor occupancy increases further, inducing a greater release of inhibitory α_1 subunits of melatonin GPCRs. These subunits increasingly bind to and inhibit adenylyl cyclase resulting in an inhibition of intracellular cAMP accumulation during the dark phase "Fig. (5)". As melatonin levels peak near the mid-dark phase, cyclic AMP-dependent FATP activity decreases to a minimum resulting in the obstruction of

linoleic acid uptake and 13-HODE formation. As a result, tumor cell proliferation decreases substantially because much

less 13-HODE is available for positive feedback on the MEK/MAPK signaling cascade and enhancement of EGF-

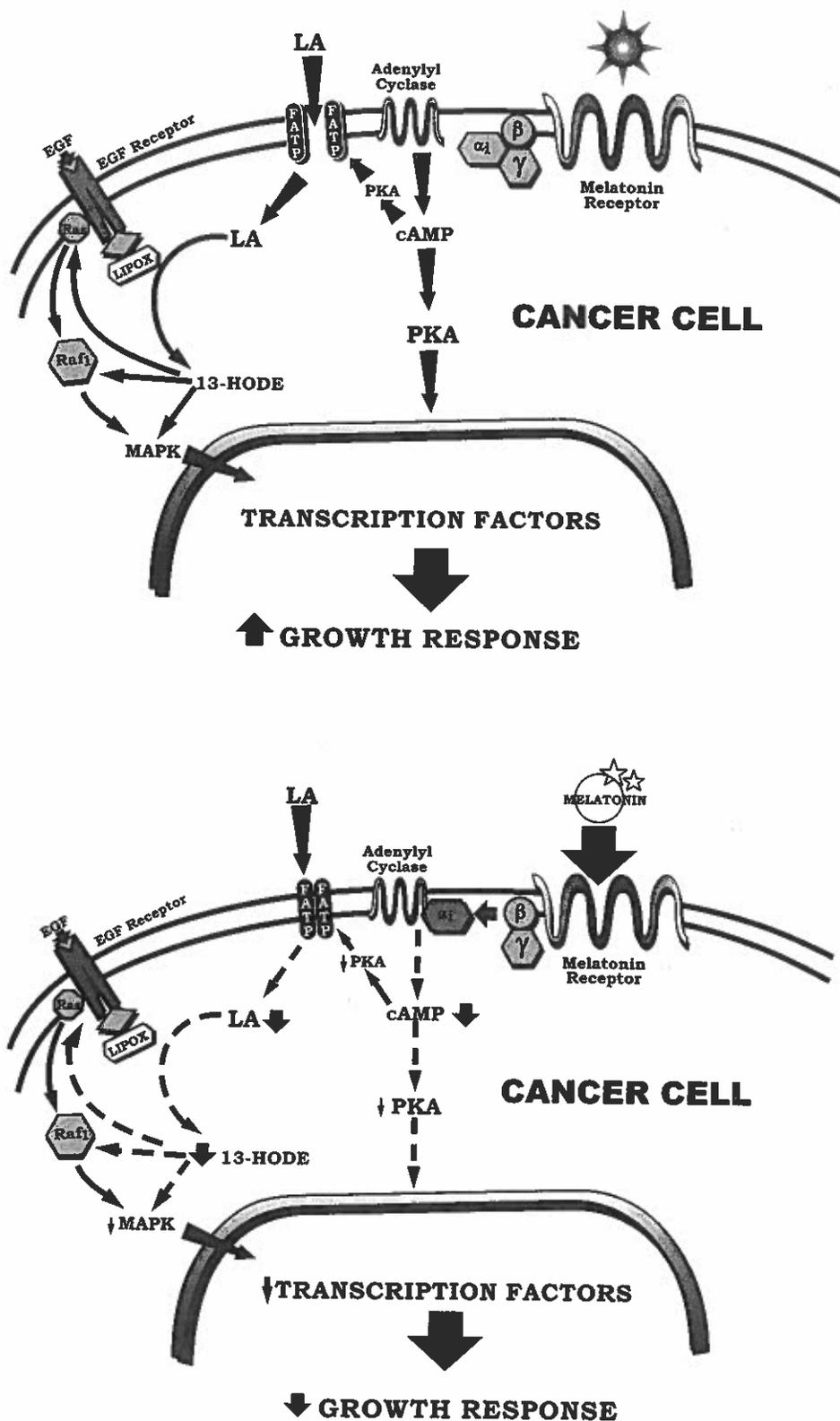


Fig. (5). Provisional diagrams for the proposed signal transduction mechanism that mediates control of tumor linoleic acid uptake (LA), 13-hydroxyoctadecadienoic acid formation and tumor growth in hepatoma 7288CTC. Daytime growth stimulation is represented in the upper diagram and melatonin-induced growth inhibition is depicted in the lower diagram.

induced mitogenic signaling. As darkness progresses, high, but decreasing melatonin levels begin down-regulating tumor melatonin receptors until both circulating melatonin titers and tumor melatonin receptors reach their lowest values by the onset of the next light phase; the cycle then repeats itself. In a sense, the tumor is "awake" during the day and growing while at night melatonin puts tumor metabolism and growth to "sleep".

Treatment of tumor-bearing rats with late afternoon melatonin injections extends the duration of high circulating melatonin levels which then summate with the endogenous melatonin rise thus creating a chemically-induced "long night" [4]. These high, late afternoon blood levels of melatonin would be coincident with late afternoon, up-regulated tumor melatonin receptors and cause an increased inhibition of linoleic acid uptake and 13-HODE formation and suppression of tumor growth. On the other hand, in pinealectomized or constant light-exposed tumor-bearing animals, even though tumor melatonin receptors would be constantly up-regulated throughout the day, the lack of circulating melatonin would allow tumor linoleic acid uptake and metabolism to proceed unchecked, "24/7", resulting in accelerated tumor growth.

Other linoleic acid uptake/EGF-related mechanisms by which melatonin could inhibit cell proliferation and tumor growth might include an inhibition of the ability of 13-HODE and other hydroperoxides of linoleic acid to suppress gap junctional intercellular communication. This could be accomplished via inhibition of linoleic acid uptake and/or via melatonin's free radical scavenging properties to protect gap junctions from free radical damage induced by linoleic acid-derived hydroperoxides. Another mechanism might involve melatonin-induced suppression of efficient DNA repair in actively growing tumor cells. Interference with EGFR signaling is thought to impair DNA repair activity mediated by DNA-PK [78]. Melatonin, through its ability to subvert the EGFR/MEK/MAPK signaling could prevent tumor growth by inhibiting 1) EGF-suppressed apoptosis [79], 2) EGF-induced stimulation of angiogenic factors such as vascular endothelial growth factor (VEGF) [79], and/or 3) EGF-mediated cytoskeletal reorganization in preparation for and during cell division [80]. Thus, melatonin suppression of the EGFR/MEK/MAPK signaling cascade via the blockade of linoleic acid uptake and 13-HODE production forms the basis for a unifying and testable model that attempts to explain melatonin's ability to inhibit tumor growth in a chronobiological context. This provides a new understanding of the multifrequency nature and integration of the environmental influences of dietary fat, the photoperiod (as represented by the melatonin signal), and the circadian system with systemic, cellular, biochemical and molecular pathways in the maintenance of the host/cancer balance [79].

Implications of Melatonin-Induced Suppression of EGFR-Mediated Mitogenic Signaling in Chronobiologically-Based Cancer Therapy

Over the past 30 years there have probably been nearly 50 small clinical trials that have been performed to test

melatonin's clinical efficacy in cancer patients. The overwhelming majority of these trials have been performed and published by a clinical oncologist, Dr. Paolo Lissoni, and his colleagues from the Division of Radiation Oncology, S. Gerardo Hospital in Monza, Italy. These trials have been reviewed in great detail in previous publications to which the reader is referred [7,81,82]. In a few cases, melatonin was given as a single agent while in the majority of studies, it was administered in combination with other therapies, including chemotherapy or radiation therapy, to patients with advanced stage malignancies. These trials consisted of primarily of non-randomized broad phase II trials in which melatonin therapy was administered to patients with a wide range of malignancies whereas a few trials were randomized, controlled phase II trials that were disease-specific for lung cancer, colorectal cancer, breast cancer, glioblastoma and brain metastases. In most of these trials, an oral dose of anywhere from 10 – 50 mg of melatonin (usually 20 mg) was administered to cancer patients in the early evening either alone or concurrently with chemo- or radiation therapy. Although an objective partial tumor response was observed in a small percentage of patients receiving melatonin, the majority of tumor responses consisted of disease stabilization. Probably the most dramatic effect of melatonin treatment was a markedly improved 1-yr survival in these patients compared with those receiving supportive care or chemotherapy alone [82].

Other benefits that accrue from melatonin therapy as reported in Lissoni's studies are a reduction in the toxicities associated with chemotherapy including myelotoxicity, nephrotoxicity, thrombocytopenia, lymphocytopenia, stomatitis, neuropathy and cancer cachexia. Probably, the most clinically important aspect of these trials is that melatonin-treated cancer patients apparently achieve and maintain better performance status and experience less anxiety than those individuals not receiving the indoleamine. Performance status represents the results of subjective assessment of the amount and timing of daytime and nighttime activity and daytime and nighttime rest or sleep. In fact, patients who have a better performance status at the outset, usually respond more favorably to melatonin alone or in combination with other more conventional therapies. Performance status has been shown repeatedly to be predictive of survival, quality of life, response to a variety of therapeutic approaches including surgery, chemotherapy, hormonal therapy and/or radiation [82].

The most prominent and bothersome universal symptom in advanced cancer patients is fatigue. There is no question that oral melatonin administration effectively increases serum concentrations that exert circadian stage-dependent biological effects on body temperature, sleep induction, circadian organization and the host/cancer balance. However, it is problematic exactly what time of day is optimal for the administration of melatonin to cancer patients to achieve its most efficacious anticancer effects as outlined above. Such trials are conspicuous by their absence in the melatonin clinical cancer literature. This is indeed surprising, if not disconcerting, since melatonin is a chronobiotic "extraordinaire" and it has been clearly shown that optimal circadian timing of non-chronobiotic, conventional cancer

therapies results in increased efficacy and decreased toxicity [82].

Although we know that melatonin administered at one particular time of day (\approx 8 PM) appears to improve the efficacy and reduce the toxicity of chemotherapy, we do not understand the biological basis for these effects. Based on the evidence presented above concerning melatonin's ability to inhibit tumor growth by suppressing the EGFR/MEK/MAPK mitogenic signaling cascade via a circadian stage-dependent, melatonin receptor-mediated blockade of linoleic acid uptake, it is our belief that we may have fortuitously uncovered a central mechanism by which melatonin improves the responsiveness of cancer patients to cytotoxic chemotherapy and radiation therapy. This proposition is based on exciting new basic science and clinical research findings demonstrating that RTKs, as exemplified by EGFR, that are mutated or overexpressed, play a critical role in malignant transformation and in supporting cancer growth [79]. Members of the EGFR family, including EGFR and Her2, are overexpressed in a variety of human cancers including non-small cell lung cancer (NSCLC) and glioblastoma and their presence is associated with a poor prognosis and resistance to cytotoxic chemotherapy [79,83]. Therapeutic strategies that inactivate growth factor receptors or their downstream pathways (i.e., MEK/MAPK) prevent tumor growth in the experimental setting. However, in the clinical arena, the blockade of the receptor binding domain with monoclonal anti-EGFR antibodies or inhibition of the tyrosine kinase domain with small molecular weight kinase inhibitors has resulted in only modest success. Nevertheless, great excitement has been generated by the combined use of inhibitors of growth factor receptors with cytotoxic chemotherapy or radiation treatment because inhibition of the RTK/MEK/MAPK pathway enhances the efficacy of these therapies.

Although complex and not fully understood, growth factor stimulation of this pathway favors cancer cell survival and proliferation. This is achieved by raising the threshold for apoptosis in response to chemotherapy and radiotherapy-induced DNA damage and also by stimulating angiogenesis [79,84,85]. In this regard, increased dietary intake of linoleic acid and/or its increased release from endogenous fat stores, coupled with increased tumor uptake could have the same effect via 13-HODE-induced phosphorylation and activation of the RTK/MEK/MAPK pathway, particularly in those tumors that overexpress these RTKs and FATP. Chemotherapeutic agents such as cisplatin as well as radiation therapy induce DNA damage that paradoxically triggers MAPK activation and antagonism of cell death. Pharmacological interruption of the RTK/MEK/MAPK pathway effectively blocks chemotherapy and radiation therapy-mediated MAPK activation and increases the lethality of these treatments [84,85].

Melatonin, either produced endogenously at night or exogenously supplied at the appropriate circadian stage, could act as an indirect kinase inhibitor by its ability to block linoleic acid uptake and 13-HODE formation and thereby decrease cell proliferation and increase cell differentiation and apoptosis via such a mechanism. Melatonin inhibition of this pathway could also decrease

angiogenesis inasmuch as this indole decreases VEGF levels in patients with advanced stage cancer [86]. Melatonin-induced interruption of the RTK/MEK/MAPK pathway could also act in concert with its free radical scavenging and antioxidant effects in reducing DNA damage in cancer cells. As alluded to above, induction of DNA damage in cancer cells by chemotherapy or radiation therapy paradoxically promotes MAPK activity resulting in suppression of apoptosis. By virtue of its free radical scavenging activity, melatonin could theoretically protect cancer cells from DNA damage and together with its anti-RTK activity, lower the threshold for chemotherapy- and radiotherapy-mediated lethality. At the same time melatonin could also protect normal tissues expressing EGFRs from being sensitized to the cytotoxicity of these therapies via its free radical scavenging/antioxidant properties. We have in fact observed such a phenomenon in rats bearing hepatoma 7288CTC in which late afternoon melatonin treatment, but not morning therapy, makes these tumors more sensitive to low dose radiation while at the same time protecting adjacent normal tissues from radiation induced toxicity [87].

What has been discussed above forms the first plausible mechanistic explanation for Lissoni *et al.*'s observations of melatonin-induced enhancement of chemosensitivity and radiosensitivity with a concomitant reduction in toxicity in NSCLC and head/neck cancer patients, and glioblastoma patients, respectively [88,89]. Additionally, melatonin GPCR-mediated inhibition of lipolysis in white adipose tissue in tumor bearing rats [90], as well as its suppression of TNF α levels in cancer patients [91], may also provide a mechanistic basis for its clinically apparent anticachectic effects as well. Thus, melatonin's considerable potential as a new anticancer hormone/drug, which may not be particularly obvious as single agent, may only be fully realized in circadian-based, combination trials with chemotherapy, hormonal therapy and/or radiotherapy as Lissoni's work would suggest. Such circadian-based combination trials with melatonin are currently under development here in the United States.

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