

# The Effect of Melatonin on Mast Cell Activation

An honors thesis for the Department of Biology

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Table of Contents

Abstract.....	3
Introduction.....	4
Materials and Methods.....	14
Results.....	20
Discussion.....	27
Works Cited.....	34

## **ABSTRACT**

Mast cells are a heterogeneous population of inflammatory cells derived from hematopoietic stem cells. Located in nearly all tissues of the body, mast cells have been implicated in allergic disorders, tissue remodeling, wound healing, and immune modulation. Upon activation, mast cells release a variety of both preformed and *de novo* synthesized chemical mediators through a process known as degranulation. These mediators have been found to have both positive and negative regulatory effects on the immune system, often benefiting the body, but sometimes causing damage. Melatonin is a hormone synthesized primarily in the pineal gland in the brain. It is best known for the regulation of circadian rhythm and its light-dependent synthesis. Melatonin has also been implicated in many of the same diseases as mast cells, including Alzheimer's Disease and many types of cancer, and has shown to be an effective immune regulator in certain systems. Rat mast cells have been shown to express both isotypes (MT1 and MT2) of the melatonin receptor on their cell surfaces.

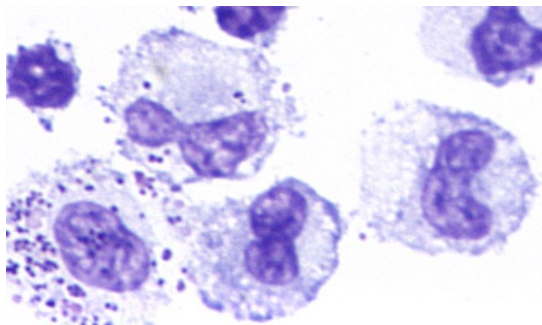
I hypothesized that HMC-1 human mast cells expressed these same receptors on their cell surface. In addition, I hypothesized that melatonin would interact with these mast cells, causing some change in the degree of mast cell activation. Results from this study not only showed that HMC-1 human mast cells express mRNA messages for MT1 and MT2, but also showed that HMC-1 and rat mast cell activation seem to be partially inhibited by incubation with melatonin. Better understanding of this modulation in mast cell activity could have an impact on the way mast cell interactions are understood and how melatonin is used as a clinical intervention in patients suffering from chronic inflammation.

## INTRODUCTION

### ***Mast Cell Activity***

Mast cells have long been regarded as key effector cells in IgE-associated immediate hypersensitivity and allergic disorders, as well as in certain protective immune responses to parasites (1). Located in nearly all tissues of the body, they have also been implicated in wound healing, tissue modeling and repair, and homeostasis. Additionally, mast cells play critical roles in both innate and adaptive immunity, including the development of immune tolerance (2). For example, mast cells, filling both sentinel and effector roles, have been shown to have net effects favoring the host in certain parasitic infections and help enhance host resistance to several types of bacterial infection (3).

However, alterations in normal mast cell behavior could have implications in the pathogenesis of autoimmune disorders, cardiac diseases, cancer, and other chronic inflammatory conditions (2,4). While the inflammatory response is homeostatic in principle, it can become pathobiologic when the same pathway leads to an outcome that is more detrimental than beneficial to the host (5). Mast cells are increasingly viewed as versatile effector and immunoregulatory cells that occupy a critical position at the interface of innate and acquired immunity. Depending on the setting, including the severity, type of infection, or the presence of another disorder, mast cells can function as either positive or negative immunoregulatory cells of both adaptive and acquired immune responses, thereby promoting health or increasing pathology during host response to an antigen (3).



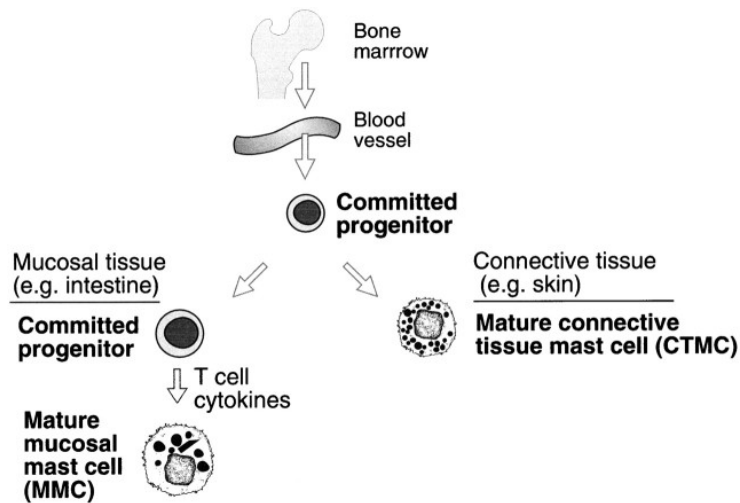
**Figure 1.** Toluidine blue stained HMC-1 human mast cells. Blue granules indicate the protein heparin (Samoszouk M, Kanakubo E, Chan J. 2005. *BMC Cancer*. 5:121).

### ***Mast Cell Development***

Like leukocytes, mast cells are derived from hematopoietic stem cells. However, unlike the cells of other hematopoietic lineages, they do not circulate in their mature form and do not dominate a single organ like parenchymal cells (3,5). Instead, mast cell precursors formed in the bone marrow during hematopoiesis circulate as committed progenitors and only mature upon entering peripheral tissues (1,3,5,6). It is in these peripheral tissues that mast cells mature into phenotypically distinct populations in different anatomical sites (1).

Mast cells are widely distributed throughout vascularized tissues, particularly near surfaces exposed to the external environment, including the skin, airways, and gastrointestinal tract. Because of their residency in these tissues, mast cells are well positioned to be one of the first cells in the immune system to interact with environmental antigens, environmentally derived toxins, or invading pathogens (3). The presence of these cells in peripheral tissues depends on the action of their cell surface tyrosine kinase, *c-kit*, and its ligand, stem cell factor (SCF) (5). Stem cell factor (sometimes known as *kit* ligand) is the main survival and developmental factor for mast cells. However, many other growth factors, cytokines, and chemokines can also influence mast cell numbers and phenotype, including IL-3, Th2-associated cytokines, and TGF- $\beta$ 1 (3).

Mast cells are also known to be potentially long-lived cells that can re-enter the cell cycle and proliferate locally after appropriate stimulation (1,3). Depending on the setting, local expansion of mast cell populations may occur by several processes in addition to proliferation. Other processes of mast cell population expansion include increased recruitment to the site of mast cell activation, long-term survival of existing mast cells, and local maturation of mast cell progenitors (3).



**Figure 2.** Mast cell development and diversity. Mast cell lineage progenitors arise in the bone marrow, circulate through the vasculature, and move into tissues to complete their development (5).

### ***Mast Cell Activation and Inflammatory Mediators***

Mast cells are equipped with a large repertoire of cell-surface receptors, enabling them to interact both directly and indirectly with pathogens and environmental toxins. Upon activation, mast cells possess the ability to secrete a plethora of mediators—both preformed and newly synthesized—which serve to alert the immune response or amplify an existing response. This large variety of mediators that these cells secrete affects a broad spectrum of physiologic, immunologic, and pathologic processes (2,6).

Mast cells constitutively express on their surface substantial numbers of FcεRI, the high-affinity receptor for IgE. The number of surface FcεRI is known to be positively regulated by ambient concentrations of IgE (3). Crosslinking of FcεRI-bound IgE with bi- or multi-valent antigen initiates the activation of mast cells by promoting aggregation of FcεRI at the cell surface, resulting in downstream events that initiate a complex secretory response (1,3). Upon activation, there are two possible outcomes: the release of preformed chemical mediators stored in granules and/or the *de novo* synthesis and secretion of mediators. While some activating

signals induce both degranulation and cytokine production, others only induce the latter response (2).

The extracellular release of preformed mediators stored in the cells' cytoplasmic granules occurs through a process known as degranulation, which involves the fusion of the cytoplasmic granule membranes with the cell's plasma membrane. Degranulation can occur by two modes; classical anaphylactic degranulation, in which the entire contents of each granule are released immediately upon activation, and piecemeal degranulation, in which partial degranulation occurs and granule contents are released in a slow, progressive manner (1,2). Granule associated mediators that can be released immediately upon activation include neutral proteases, histamine, proteoglycans, vasoactive amines, heparin, and some cytokines (such as TNF- $\alpha$ ) and growth factors. Other mediators, which are synthesized *de novo* and take longer to secrete, include proinflammatory lipid mediators, such as prostaglandins and leukotrienes, and various cytokines, chemokines, and growth factors (1-3).

Mast cell populations in different anatomic sites differ significantly in the types and amounts of allergic mediators they contain (6). Mediator content in particular populations, as well as other aspects of their phenotype such as the ability of the cell to respond to particular stimuli (or pharmacological inhibitors) of activation, can be modulated, in at least some cases reversibly, by cytokines, growth factors, and other environmental signals that bind to non-Fc $\epsilon$ RI receptors expressed by this cell type (1,3). It is this activation modulation and differential release of mediators that allows mast cells the possibility of being potentially harmful instead of helpful. Abundant evidence from both mice and human indicates that antigen and IgE-dependent activation of mast cells is critical for the pathophysiological manifestations and mortality associated with IgE-dependent anaphylaxis. Additionally, it has been shown that genetic

predispositions to produce larger or smaller amounts of tumor necrosis factor (TNF) and other cytokines, or the presence of other abnormalities in the host, may also influence whether the role of mast cells is either beneficial or harmful (3).

### ***Mast Cells and Cancer***

Conditions of chronic inflammation stimulate proliferation of resident tissue mast cells and promote the local recruitment of circulating mast cell precursors. Much research has been done recently looking at this behavior in relation to the pathogenesis of many types of cancer. During tumor development, mast cells form one of the major inflammatory cell populations and are now considered critical regulators of inflammation and the immunological response in the tumor microenvironment (7). In fact, mast cell infiltration in a tumor has become an independent prognostic factor and predictor of poor outcome in some cancers, including Hodgkin lymphoma, Merkel cell carcinomas, and prostate, colorectal, lung, thyroid, and pancreatic cancers (8,9). Because of this close association of mast cells with tumors, and the apparent capacity of mast cells to promote tumor proliferation and invasion both directly by stimulating tumor cells and indirectly by modulating the tumor microenvironment, mast cells appear to have a central role in this pathologic developmental process (8,9).

In tumors, mast cells are recruited and activated via several factors secreted by tumor cells. Perhaps most important (and most well studied) of these factors is SCF (7). Past studies have shown that tumor cells generally produce SCF, and that this tumor-derived SCF can be responsible for the maturation of sentinel tissue-associated mast cells and the recruitment of circulating mast cell progenitors (8,9). Mast cells have been observed to infiltrate enlarging cancerous growths and the invasive front of carcinomas, but not the core of solid tumors (10). It



is now quite certain that mast cell infiltration to tumors is increased in comparison with normal tissues and accordingly appears to be able to exert a protumor effect (8).

One of the major issues linking mast cells to cancer is the ability of these cells to synthesize and release pro-angiogenic factors. Angiogenesis, or the formation of new vessels from pre-existing ones—such as capillaries and post-capillary venules—plays a pivotal role in tumor development, providing nutrients necessary for cell growth. In addition, angiogenesis is a necessary step for the metastasis of solid tumors to distal sites of the body. The association of mast cells and new vessel formation has been reported in many types of cancer, including breast cancer, colorectal cancer, and uterine cervical cancer (7,8,10). Mast cells infiltrate into the boundary between normal tissues and tumors and express many pro-angiogenic compounds and were seen to degranulate in close apposition to capillaries and epithelial basement membranes (7,8,10). Some of these released pro-angiogenic mediators include fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF), heparin, histamine, IL-8 and various proteases (7,9,10).

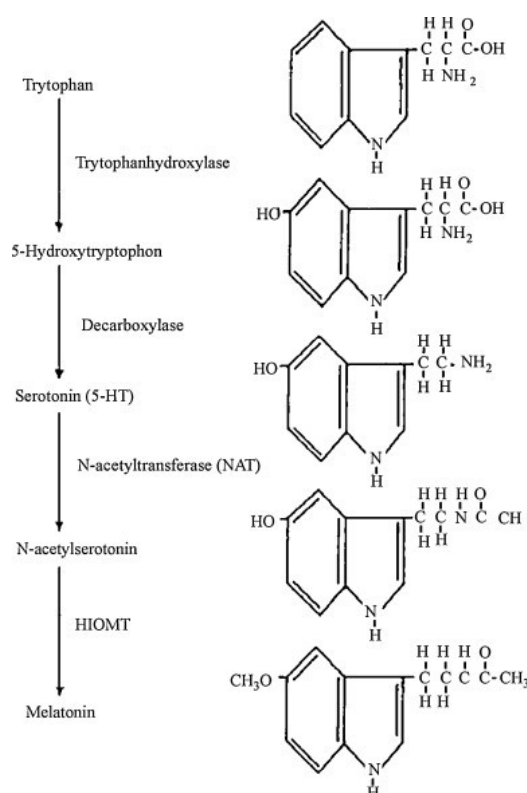
In addition to this angiogenic activity, mast cells can directly influence tumor cell proliferation and invasion but also help tumors indirectly by organizing its microenvironment and modulating immune response in tumor cells (9,10). It has been suggested that in the context of developing tumors, the ability of mast cells to remodel healthy tissues is subverted. Instead, these mast cells disrupt the surrounding extracellular matrix (ECM) and aid in increasing tumor spread. In addition to providing room for tumor growth, the disruption of local ECM leads to the release of matrix-bound factors including SCF, thereby increasing endothelial cell migration and proliferation and likely promoting further angiogenesis, tumor spread, and growth (10). Mast cells are also indispensable in orchestrating innate and adaptive immune responses to promote

cancer, releasing histamine, adenosine, TNF- $\alpha$  and other immune regulating cytokines such as IL-6, IL-10, and IL-23, all of which promote immune tolerance and down-regulation of immune response, especially by T regulatory cells at the site of tumor development (7-9). Mast cells therefore seem to be capable, either independently or synergistically, of promoting proliferation, survival, and severity of many types of cancer cells.

### ***Melatonin***

Melatonin (*N*-acetyl-4-methoxytryptamine) is a hormone product of the amino acid tryptophan and is chiefly secreted from the pineal gland in the brain. Though initially believed to be restricted to the pineal gland, melatonin synthesis has in fact been observed in many other organs, including the retina, the gut, and in bone marrow-derived cells such as mast cells (11). Most commonly, it is known for its involvement in the regulation of human circadian rhythm and sleep/wake cycles. In addition to serving this function, melatonin also acts as a direct free radical scavenger, an indirect antioxidant, and an immunomodulatory agent (11). This hormone has the ability to reduce macromolecular damage to many tissue types by scavenging free radicals such as the hydroxyl radical, peroxynitrite radical, and hypochlorous acid that would otherwise initiate an inflammatory response. Melatonin also has been shown to prevent the activation of NF- $\kappa$ B (a transcription factor responsible for the production of many pro-inflammatory mediators such as IL-2, IL-6, and TNF- $\alpha$ ), most likely by hindering the translocation of the transcription factor across the nuclear envelope (12). Additionally, melatonin affects the T-cellularity of mice spleens (which usually contain high numbers of T-cells) by reducing spleen weight when endogenous melatonin is inhibited (thereby decreasing the number of T-cells present) and is counteracted with subsequent melatonin administration (11). This data clearly implicates melatonin in the regulation of inflammation and immunity.

This connection between melatonin and inflammatory responses spurred further investigation into melatonin's relation to specific inflammatory diseases. Significant reductions in plasma concentrations of melatonin were observed to be associated with headache, coronary heart disease, chronic pain, and Alzheimer's disease, all of which have at least some inflammatory component of their pathology (11). In addition to these, there is growing evidence that supports melatonin's involvement in cancer pathogenesis and disease progression. For example, in human melanoma cells, melatonin was proven to be relatively potent in suppressing cancer cell proliferation after 6 days of culture (11). In another recently published long-term study, a correlation was found between the number of years nurses worked overnight shifts in a hospital and an increased risk of developing colorectal cancer (13). This is likely due to the nature of melatonin synthesis in humans. In normal human populations, daytime plasma levels of melatonin are low and reach maximal levels during the night. However, exposure to light of the appropriate intensity and wavelength (such as those that illuminate hospitals) can disrupt melatonin production, resulting in abnormally low plasma concentrations of the hormone (11,12).



**Figure 3.** Biosynthetic pathway of melatonin and enzymes implied (18).

### ***Melatonin Receptors***

Melatonin receptors in mammals are classified as membrane-bound, high-affinity G-protein coupled receptors. All of melatonin's cellular actions are likely exerted by two known G-protein coupled receptor isoforms, denoted MT1 and MT2. Both receptors share a high degree of homology and are expressed at low levels in numerous organs and cell types (14,15).

These receptors are known to interact with inhibitory G-proteins of the Gi2 and Gi3 classes. Upon ligand binding, the Gi-linked signaling pathway is initiated, leading to the inhibition of adenylate cyclases and, subsequently, to the reduced levels intracellular cyclic adenosine-monophosphate (cAMP), diminished protein kinase A (PKA) activation and, consequently, to changed phosphorylation of cAMP responsive element binding, as well as effects on adenyl cyclases, phospholipase A2 and C, and calcium and potassium channels. This signaling cascade thus ultimately may result in altered gene transcription, leading to changes in downstream cellular activity (14-16).

In addition to melatonin, both MT1- and MT2-receptors have been shown to specifically bind no less than 32 and 14 other proteins respectively (15). This presents the opportunity for the existence of receptor agonists and antagonists. In a murine model of hemorrhage and resuscitation, the melatonin receptor antagonist luzindole was able to attenuate the protective effects of melatonin pretreatment and therapy with respect to liver function. In the same model of hemorrhagic shock, therapy with the selective melatonin receptor agonist ramelteon improved liver function and hepatic perfusion in rats (14). Another study showed that the anti-invasive cancer cell response to melatonin was enhanced by overexpression of the MT1 receptor and inhibited by the administration of luzindole, thereby demonstrating that the

antimetastatic effects depend on the binding of melatonin to its specific membrane receptors (17). Further implicating melatonin receptors as a necessity for the beneficial activity of melatonin, in a murine model of sepsis, improvements in survival seen after melatonin therapy were not present in melatonin receptor double knock-out mice, showing that the absence of melatonin receptors directly impedes the protective action of melatonin administration (14).

### ***Mast Cells and Melatonin***

Recently, mast cells have been added to the list of sources outside of the pineal gland where melatonin can be synthesized and released. A recent study in rats showed that rat mast cells not only expressed both MT1 and MT2 receptors on the cell surface, but also showed activity in two key enzymes in the production of melatonin, N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT). This may indicate that mast cell activation could lead to melatonin synthesis and secretion which, when paired with the MT1 and MT2 receptors, may indicate cell participation in a melatonin-dependent feedback loop (18). Another study showed that in a model of water avoidance stress-induced degranulation of mast cells in the dermis of rats, chronic melatonin treatment reduced water avoidance stress-induced infiltration and activation of mast cells in the dermis (19). It should also be noted that low melatonin levels have been implicated in many diseases, including Alzheimer's Disease and certain cancers (16, 20-22). Given the previously mentioned implication of mast cells in these and similar diseases, it would logically follow that there may be some direct inhibitory interaction between melatonin and mast cells. In this current study, the possibility of this interaction was explored by confirming the presence of melatonin receptors on the surface membrane of HMC-1 human mast cells as well as investigating the potentially inhibitory effect of melatonin on mast cell activation and degranulation in both human HMC-1 and rat mast cell lineages.

## **MATERIALS AND METHODS**

### ***Culture of Human Mast Cells***

Human mast cells of the HMC-1 lineage were kindly provided from Dr. Paul Butterfield (Mayo Clinic) and cultured in 10 mL Hyclone Iscoves Modified Growth Media containing 5% fetal calf serum (FCS). Cells were grown in a humid atmosphere containing 5% CO<sub>2</sub> at 37° C and were split using sterile technique 1:4 every 4-5 days. Cells that had gone through 10 or more passages were not used in these experiments in order to ensure their livelihood and natural function.

The mast cell leukemia derived HMC-1 human mast cell line is one of very few human mast cell models used for experimentation. However, unlike mature mast cells, HMC-1 cells only variably express the  $\alpha$ -subunit of the Fc $\epsilon$ RI complex. As a result, they are known to inconsistently degranulate to IgE-dependent signals. Additionally, they are growth factor independent, not needing SCF to survive due partially to a mutation in the SCF receptor. Therefore, they phenotypically resemble immature mast cells more so than healthy mature mast cells (23,24). This being said, they do express low levels of most mast cell markers relative to mature mast cells, and therefore can be used as a limited model for human mast cell experimentation (25).

### ***RNA Extraction and RT-PCR***

In order to identify an mRNA message for melatonin receptors in human mast cells, RT-PCR and gel electrophoresis was used. RNA was extracted from HMC-1 cells by first centrifuging cells (1500 rpm for 4 minutes) to remove culture media. The cells were then resuspended in 1 mL TRI Reagent (Molecular Research) per 10<sup>7</sup> cells, allowed to sit at room

temperature for 5 minutes, and 0.1 mL of 1-bromo-3-chloropropane (BCP) added for every 1 mL of TRI Reagent used. Cells were then vortexed and allowed to sit at room temperature for another 15 minutes. The samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C. The aqueous phase (not containing DNA and protein) was then transferred to a fresh tube. RNA was precipitated by adding 0.5 mL isopropanol for every 1 mL of TRI Reagent in the starting solution. Samples were stored at room temperature for 10 minutes before centrifugation at 12,000 rpm for 8 minutes at 4°C. The supernatant was then removed and the remaining pellet was washed with 2 mL of 75% ethanol for every 1 mL of TRI Reagent in the starting solution. Samples were vortexed and centrifuged again at 12,000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet was allowed to air-dry for 3 minutes. The pellet was then re-solubilized in 50 µL RNA storage buffer (Ambion) and stored at -80°C until needed.

DNase treatment (used to remove any remaining DNA and ensuring no molecular contamination), reverse transcription, and PCR were performed using reagents provided by Ambion according to manufacturer's instructions. Sense and antisense primers for both the MT1 and the MT2 melatonin receptors (Sigma-Genosys) were used to amplify the corresponding regions in the HMC-1 derived transcript. In order to separate DNA fragments by size, the samples were then subjected to gel electrophoresis, running at 110 volts on a 1.5% agar gel containing 10 µL 1 mg/mL Ethidium Bromide. A 100 base pair DNA ladder was used for reference. After the bands of cDNA had run approximately 75% of the length of the gel, the gel was rinsed with deionized water and analyzed under UV light. A picture of the gel, exposed under UV light, was taken using a Kodak DC290ZOOM camera and imaging software (Kodak).

### ***HMC-1 Treatment with Melatonin***

Prior to incubation, a 100 mM solution of melatonin (Sigma) was prepared by dissolving 23 mg of melatonin in 1 mL DMSO. This solution was then diluted with Hank's Solution buffer containing 1 mg/mL bovine serum albumin (BSA) with a pH of approximately 7.4 to produce a 10 mM melatonin solution.

In order to incubate cells with melatonin, equal volumes of HMC-1 cell suspension were transferred to a 6-well plate. To test for a concentration-dependent inhibition, an appropriate volume of the 10 mM melatonin solution was added to each well to yield a set of experimental samples that had final melatonin concentrations of 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, and 200  $\mu$ M (depending on the experiment). The cells were then allowed to incubate for 20 minutes before moving on to the  $\beta$ -hexosaminidase assay in which mastoparan or neurotensin was used to stimulate the cells.

To test for time-dependent inhibition, an appropriate volume of 10 mM melatonin solution was added to the cells in culture media to produce a final melatonin concentration of 100  $\mu$ M. The melatonin solution was added at various time points to individual wells in order to yield a set of experimental samples that had been incubating with melatonin for 5 minutes, 10 minutes, 15 minutes, 20 minutes, and 30 minutes before moving on to the  $\beta$ -hexosaminidase assay, again using either mastoparan or neurotensin to activate the mast cells.

### ***$\beta$ -hexosaminidase Release Assay***

$\beta$ -hexosaminidase is an enzymatic component of mast cell granules, the activity of which can be readily monitored by a relatively sensitive colorimetric assay (26). This method was used as an indicator of the level of stimulation of the mast cells. To begin, cultured HMC-1 cells were prepared for the assay by first removing them from their FCS culture media by centrifugation at room temperature (5 minutes at 1500 rpm). The cells were then resuspended in



Hank's Solution containing 1 mg/mL bovine serum albumin (BSA) with a pH of approximately 7.4 to yield cell suspensions containing 50,000-300,000 HMC-1 cells/mL. Actual cell concentration was determined using a Hausser Hi-Lite Ultra Plane Hemocytometer.

A 200  $\mu$ L volume of cell suspension was placed into individual glass test tubes and incubated at 37°C for 15 minutes. 200  $\mu$ L of the stimulus, either mastoparan, a protein derived from wasp venom (Sigma) or neurotensin (Bachem), a neuropeptide and known mast cell activator (27), was then added to the cells at a final concentration of 100  $\mu$ M. An equivalent volume (200  $\mu$ L) of Hank's Solution was added to control samples containing no stimulator. The cells were allowed to incubate for an additional 20 minutes at 37°C.

After incubation, the cells were separated from the buffer solution by centrifugation at room temperature (5 minutes at 1800 rpm). The supernatant containing any released  $\beta$  - hexosaminidase was then poured off into fresh test tubes. To the cell pellet fractions, one volume of Hank's Solution equivalent to the volume of the supernatant fractions (400  $\mu$ L) was added. A sonicator was then used to lyse the cells in the cell pellet fraction in order to liberate any remaining  $\beta$  -hexosaminidase present in the cells. At this point, the protocol could be halted by freezing samples at 20°C until needed. If frozen, samples were allowed to thaw completely and were vortexed before continuing.

A 50  $\mu$ L volume of each supernatant fraction or cell lysate fraction was then added to an individual well of a 96-well plate. Then, 100  $\mu$ L of 2.5 mM p-nitro (p-nitrophenyl-N-acetyl B-D glucosaminide) in 0.04 M citrate buffer with a pH of 4 was added to each well. The plate was gently vortexed to ensure even mixing, and then wrapped tightly with aluminum foil to prevent evaporation. The plate was then incubated with gentle shaking at 37°C for 90 minutes.

After incubation, the plate was removed and 50  $\mu$ L of 0.4 M glycine was added to each well. The plate was then gently vortexed to ensure adequate mixing of all reagents. After allowing the samples to cool to room temperature (approximately 5 to 10 minutes), the plate was read by a Bio-Rad Benchmark Plus Microplate Spectrophotometer at a wavelength of 405 nm with a reference at 570 nm. The amount of  $\beta$ -hexosaminidase present in the supernatant was compared to the total  $\beta$ -hexosaminidase from the sample (cell pellet fraction + supernatant fraction) to ascertain the percent of  $\beta$ -hexosaminidase released. Calculating the percent release controls for the potentially different concentration of cells in each sample, allowing comparison across different treatments within the same experiment and across different experiments.

### ***Histamine Release Assay***

To explore other *in vitro* models of mast cells and potential melatonin inhibition, rat mast cells were acquired from the phenotypically distinct pleural and peritoneal cavities of three rats (28). The release of histamine, a well-known component of mast cell granules, was assayed to determine potential inhibition by melatonin in these rat mast cells.

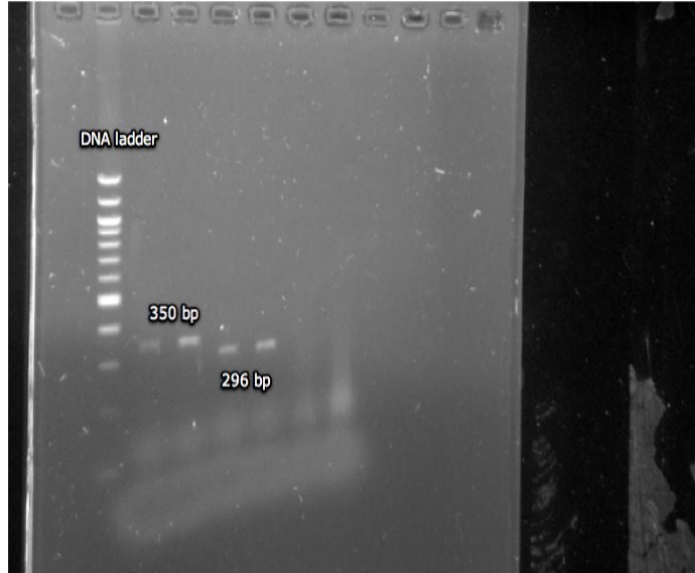
To begin, harvested cells were incubated in polystyrene tubes at 37°C for 15 minutes with 100  $\mu$ M melatonin. After this incubation period, the mast cell stimulator was added for cells were incubated at 37°C for another 15 minutes. Two mast cell stimulators at varying concentrations were used in this experiment: neurotensin (1  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, 50  $\mu$ M) and mastoparan (10  $\mu$ M, 20  $\mu$ M). It should be noted that in this experiment, the more sensitive pleural cells were incubated with 1  $\mu$ M and 10  $\mu$ M concentrations of neurotensin, while peritoneal cells or a mixed population of pleural and peritoneal cells was used for the remaining treatments. After incubation with stimulator, samples were centrifuged for 5 minutes at 2000 rpm and supernatant was poured off into a separate set of polystyrene tubes. To acidify the cell samples, and thereby stabilize the

histamine present, 0.1 mL of 20% perchloric acid was added to each supernatant and 1 mL of 2% perchloric acid was added to the cell pellets. Here, cell samples could be frozen at -20°C for later use.

Samples containing just the cell pellet were then boiled to ensure cell lysis and protein degradation. All samples (cell pellets and supernatants) were then centrifuged at 2500 rpm for 15 minutes. When finished, a 0.2 mL aliquot of each sample was removed and added to individual glass culture tubes, making sure to avoid the transfer of precipitate containing unwanted protein product and other cell material. Distilled water was added to each aliquot to bring the final volume to 2.0 mL. 0.2 mL of 2N NaOH was then added to each sample, briefly vortexed, and then 0.1 mL of 0.1% OPTA (in 100% methanol) was added and samples were vortexed again. After four minutes, 0.2 mL of 2.5 M phosphoric acid was added to each sample, halting the reaction. Fluorescence of samples were then read in a spectrofluorometer set to 364 Excitation and 415 Emission within 30 minutes of stopping the reaction. The value of the supernatant was compared to the total reading (cell pellet fraction + supernatant fraction) to determine the percent release of histamine per sample, therefore controlling for the potential for differential cell distribution amongst the samples and allowing comparison across samples and across different tests.

### ***Statistical Analysis***

Statistical significance was tested using a two sample unpaired student's t-test assuming equal variance across samples. Differences were considered to be significant at  $p < 0.05$ .



## RESULTS

### *HMC-1 cells express mRNA*

### *messages for melatonin receptors*

### *MT1 and MT2*

While previous studies have shown that rat mast cells express both isoforms of the melatonin receptor, MT1 and MT2, on their surface membrane (18), there is little published evidence confirming the presence of these receptors on human mast cells of the HMC-1 lineage. In an effort to confirm the presence of these receptors in HMC-1 cells, RNA was isolated from the cells and RT-PCR was performed using primers for the two known melatonin receptors. The resulting cDNA was then subjected to gel electrophoresis to separate the digested DNA bands by length. Upon visualization with ethidium bromide, the sample yielded two distinct cDNA fragments, one at approximately 350 base pairs and one at approximately 296 base pairs (Fig. 4). These fragments correspond, respectively, to the fragment length of the MT1 and MT2 receptors, confirming that transcription of the genes for these two receptors in cells of the HMC-1 lineage takes place under normal, unstimulated conditions.

**Figure 4.** Gel electrophoresis of RNA from HMC-1 cells using primers for two melatonin receptors, MT1 and MT2. Total RNA was isolated from HMC-1 cells and subjected to RT-PCR. cDNA samples underwent electrophoresis in duplicate to ensure consistency and visualized with ethidium bromide. Two bands were visualized, one at 350 bp and one at 296 bp. These bands correspond, respectively, to the known fragment lengths for the MT1 and MT2 melatonin receptors.

### ***Concentration-Dependent Effects of Melatonin on Human Mast Cell HMC-1 Activation***

If melatonin directly inhibits mast cell-related inflammation as hypothesized, then it follows that melatonin might have some direct inhibitory effect on mast cell activation. The activity of  $\beta$ -hexosaminidase, an enzyme component of mast cell granules, can easily be determined by a simple colorimetric assay (26). The amount of  $\beta$ -hexosaminidase released into the supernatant of an experimental sample compared to the total amount of  $\beta$ -hexosaminidase in that sample (the combined amount of  $\beta$ -hexosaminidase in the supernatant and that which remains in the cell) is an easily interpreted indicator of relative mast cell activation. Mast cells cultured in FCS were incubated in a 6-well plate with melatonin at a final concentration of either 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, or 100  $\mu$ M for 20 minutes in order to investigate the possibility of a causal relationship between the melatonin concentration and the level of inhibition. Various known mast cell stimulators were screened using a population of untreated mast cells in order to determine the best activator to use in this experiment, i.e. the activator that showed the greatest amount of  $\beta$ -hexosaminidase release above basal levels at 100  $\mu$ M concentration (Fig. 5). Ultimately in this experiment, melatonin's inhibition of activation was studied in the context of two different activators: mastoparan and neurotensin (not shown).

**Figure 5.** Screen of various known mast cell activators to determine those that stimulate the greatest  $\beta$ -hexosaminidase released.  $\beta$ -hexosaminidase in the supernatant was compared to the total  $\beta$ -hexosaminidase in the sample to determine the percent release. Results shown are the results from one independent experiment.

In mast cells incubated with melatonin and exposed to mastoparan as a stimulus, there was a trend indicating that higher concentrations of melatonin corresponded to the increased inhibition of mast cell activation, showing a gradual 0.41-fold decrease in  $\beta$ -hexosaminidase release from mast cells with no melatonin exposure to mast cells incubated with 100  $\mu$ M melatonin (Fig. 6). Interestingly, this trend was not present in melatonin-incubated mast cells

exposed to neurotensin as an activator (Fig. 7). While these trends appear to exist, the results were not found to be statistically significant for any concentration of melatonin ( $p>0.05$ ).

**Figure 6.** The concentration-dependent effect of melatonin on  $\beta$ -hexosaminidase release by HMC-1 cells in Hank's solution containing 1 mg/mL BSA when stimulated with 100  $\mu$ M mastoparan at 37° C. Cells were plated in 6-well plates and incubated with melatonin for 20 minutes before stimulation. Data are representative of fold change above basal  $\beta$ -hexosaminidase release. Results shown are the mean  $\pm$  SE of 3 independent experiments.

**Figure 7.** The concentration-dependent effect of melatonin on  $\beta$ -hexosaminidase release by HMC-1 cells in Hank's solution containing 1 mg/mL BSA when stimulated with 100  $\mu$ M neurotensin 37° C. Cells were plated in 6-well plates and incubated with melatonin for 20 minutes before stimulation. Data are representative of fold change above basal  $\beta$ -hexosaminidase release. Results shown are the mean  $\pm$  SE of 3 independent experiments.

#### ***Time-Dependent Effects of Melatonin on Human Mast Cell HMC-1 Activation***

In addition to a concentration-dependent relationship between melatonin and levels of inhibition, the possibility of time-dependent inhibition by melatonin was also explored. The same  $\beta$ -hexosaminidase assay and the same two stimulators from the previous test were used. However, for this experiment, all cells were incubated with a constant 100  $\mu$ M concentration of melatonin for various time intervals. Cells were allowed to incubate with melatonin for 5 minutes, 10 minutes, 15 minutes (only in the experiment with mastoparan as an activator), 20 minutes, and 30 minutes. Paralleling the concentration-dependent results, stimulation with mastoparan showed a trend toward increased inhibition with increased incubation time with, showing a gradual 0.16-fold decrease in  $\beta$ -hexosaminidase release over approximately 20 minutes (Fig. 8). Much like the concentration-dependent analysis, neurotensin did not show the same correlation discovered when neurotensin was used as a stimulus (Fig. 9). Once again, the data establishing these trends were not found to be statistically significant for any incubation intervals ( $p>0.05$ ).

**Figure 8.** The time-dependent effect of melatonin on  $\beta$ -hexosaminidase release by HMC-1 cells in Hank's solution containing 1 mg/mL BSA when stimulated with 100  $\mu$ M mastoparan at 37° C. Cells were plated in 6-well plates and incubated with 100  $\mu$ M melatonin for various time intervals before stimulation. Data are representative of fold change above basal  $\beta$ -hexosaminidase release. Results shown are the mean  $\pm$  SE of 3 independent experiments.

**Figure 9.** The time-dependent effect of melatonin on  $\beta$ -hexosaminidase release by HMC-1 cells in Hank's solution containing 1 mg/mL BSA when stimulated with 100  $\mu$ M neurotensin at 37° C. Cells were plated in 6-well plates and incubated with 100  $\mu$ M melatonin for various time intervals before stimulation. Data are representative of fold change above basal  $\beta$ -hexosaminidase release. Results shown are the mean  $\pm$  SE of 3 independent experiments.

### ***The Effects of Melatonin on Rat Mast Cell Activation***

In order to explore other systems in which melatonin may have an inhibitory effect on mast cells, its effects were quantified in mast cells freshly harvested from the pleural and peritoneal cavities of rats. For this test, a fluorometric histamine release assay was used. Much like  $\beta$ -hexosaminidase, histamine is another granular component of mast cells that is secreted by the cell during activation events, often by the cross-linking of Fc $\epsilon$ RI-bound IgE with antigen, but also through the activation of other membrane-bound receptors (3).

Histamine release was measured in cells incubated with 100  $\mu$ M melatonin for 15 minutes followed by 15 minutes of stimulation with different concentrations of either mastoparan or neurotensin. Because rat mast cells isolated from the pleural cavity have been shown to be quite responsive to neurotensin, and those isolated from the peritoneal cavity less so (28), pleural mast cells were exposed exclusively to the low doses of neurotensin (1  $\mu$ M and 10  $\mu$ M), while peritoneal and mixed cell populations were exposed to higher doses of neurotensin (20  $\mu$ M and 50 $\mu$ M) and all doses of mastoparan (10  $\mu$ M and 20  $\mu$ M).

The results show that in all cell populations (pleural, peritoneal, or mixed), incubation with melatonin has little to no effect on inhibiting rat mast cell histamine release when stimulated with any concentration of neurotensin (Fig. 10-12). However, incubation with melatonin did show to have some inhibitory effect on peritoneal and mixed rat mast cell populations stimulated

by different concentrations of mastoparan, showing slightly steeper changes in histamine release between cells stimulated with 10  $\mu$ M mastoparan when contrasted with cells stimulated with 20  $\mu$ M mastoparan (Fig. 11-12). The data collected was not shown to be statistically significant for any cell population or stimulator concentration. ( $p>0.05$ ).

**Figure 10.** The effect of melatonin on histamine release from rat mast cells. Cells were isolated from the pleural cavities of 2 rats and incubated with 100  $\mu$ M melatonin for 15 minutes before exposure to varying low doses of neurotensin for another 15 minutes at 37° C. Data represents the percent release of histamine from pleural mast cells. Treatments followed by (-) indicate samples that were not incubated with melatonin. Results shown are the mean  $\pm$  SE of 1 experiment conducted in duplicate.



**Figure 11.** The effect of melatonin on histamine release from rat mast cells. Cells were isolated from the peritoneal cavities of 2 rats and incubated with 100  $\mu$ M melatonin for 15 minutes before exposure to varying doses of neurotensin or mastoparan for another 15 minutes at 37° C. Data represents the percent release of histamine from peritoneal mast cells. Treatments followed by (-) indicate samples that were not incubated with melatonin. Results shown are the mean  $\pm$  SE of 1 experiment conducted in duplicate.

**Figure 12.** The effect of melatonin on histamine release from rat mast cells. A mixed cell population was used, containing cells from both the pleural and peritoneal cavities of 1 rat. Cells were incubated with 100  $\mu$ M melatonin for 15 minutes before exposure to varying doses of neurotensin or mastoparan for another 15 minutes at 37° C. Data represents the percent release of histamine from both pleural and peritoneal mast cells. Treatments followed by (-) indicate samples that were not incubated with melatonin. Results shown are the mean  $\pm$  SE of 1 experiment conducted in duplicate.

## DISCUSSION

Mast cells, with their immunomodulatory properties, often make up the front lines of sites of infection, poised at the interfaces separating the body from the environment (skin, airways, gastrointestinal lining, etc.) (3). Their role in diseases with chronic inflammatory components, such as Alzheimer's Disease and cancer, has also been well categorized (2,4). Intriguingly, the hormone melatonin has been shown to have involvement in the progression of some of these same diseases, often showing an inverse correlation between melatonin levels and disease severity (16). To date, there seems to be very few studies directly linking melatonin to

mast cells. It is known that rat mast cells indeed have melatonin receptors, and in a model of water avoidance stress-induced mast cell degranulation, melatonin did appear to have a significant inhibitory effect on rat mast cell activation (19). However, no studies could be found directly linking the modulatory effects of melatonin on a human lineage of mast cells, such as the HMC-1 human mast cells.

This study was designed to address this gap in mast cell knowledge. RNA from human mast cells of the HMC-1 lineage was used to determine if these cells expressed the mRNA messages for either of the known isoform of the melatonin receptors, MT1 and/or MT2. To investigate any direct inhibition of mast cell activation by melatonin, colorimetric  $\beta$  - hexosaminidase and fluorometric histamine assays were performed on stimulated HMC-1 and rat mast cells respectively to ascertain relative levels of mast cell activation. The results from these tests helped elucidate some novel characteristics of the mast cell/melatonin interaction.

Previous research in the Cochrane laboratory highlighted the possibility of mast cell receptors MT1 and MT2 expression in human HMC-1 mast cells. To corroborate this data, total RNA was isolated from unstimulated HMC-1 cells grown in Hyclone Iscoves Modified Growth Medium. These cells should therefore give the best representation of mast cells acting at basal levels of activity (i.e. gene translation and transcription). After RT-PCR, gel electrophoresis was used to separate amplified cDNA fragments by size. Two distinct bands of cDNA were visualized, one of approximately 350 base pairs and one of approximately 296 base pairs. These correspond, respectively, to the mRNA messages for the MT1 and MT2 melatonin receptors. While the protein end product of these messages has yet to be confirmed on the surface of HMC-1 cells, mRNA expression of these genes suggests the presence of these receptors in basal HMC-1 cells, suggesting that these cells that have the potential for interacting with melatonin.

Knowing that HMC-1 cells constitutively express RNA messages for both isoforms of the melatonin receptor, the possible direct interaction between mast cells and melatonin was subsequently investigated. As previously stated, there is little published data detailing mast cell interaction with melatonin, never mind specifically human mast cell interactions with the hormone. Because of this, the potential for basic concentration-dependent and time-dependent relationships between melatonin and mast cell activity was investigated.

In order to compare basal mast cell activity to the activated and/or inhibited state of mast cells, mast cell activators were used. Four different known activators were screened at 100  $\mu$ M concentrations: mastoparan, a toxin derived from wasp venom, cortistatin, a neuropeptide that suppresses neuronal activity, Substance P, a neuropeptide associated with pain and inflammation, and A23187, a calcium ionophore that transports calcium across cell membranes down its concentration gradient. According to the  $\beta$ -hexosaminidase assay used to evaluate mast cell stimulation levels, mastoparan appeared to promote the greatest release of  $\beta$ -hexosaminidase (a 42% increase over basal release), and therefore, was the most powerful stimulator of HMC-1 cells. To stay consistent with current and past research in the Cochrane laboratory, and to examine a different mode of mast cell activation, neurotensin, a neuropeptide and well-studied mast cell activator, was also used in subsequent experiments.

In the concentration-dependent analysis of melatonin's effect on mast cell activation, HMC-1 cells were incubated with various concentrations of melatonin (10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, and 100  $\mu$ M) and then stimulated with 100  $\mu$ M of either mastoparan or neurotensin. While the data collected did not prove to be statistically significant, some interesting trends were discovered. For cells activated by mastoparan, increasing levels of melatonin concentration appeared to depress the amount of  $\beta$ -hexosaminidase released from HMC-1 cells in

comparison to negative controls. However, cells stimulated by neurotensin did not show a similar concentration-dependent trend, showing relatively large variation in  $\beta$ -hexosaminidase release among samples, no significant stimulation in control samples, and no correlation associated with melatonin concentration. This was surprising, as neurotensin has previously been documented as being an effective rat mast cell activator (27). However, this data suggests that melatonin may in fact be having some inhibitory effect on mast cell activation, but only through select activation pathways.

Because a possible concentration-dependent relationship between melatonin and  $\beta$ -hexosaminidase was unveiled, the potential for a time-dependent relationship between melatonin incubation and mast cell activation became even more intriguing. Melatonin has a relatively short half-life *in vivo* (approximately 10 minutes) due to rapid turnover and degradation in the liver (15). Because of this, the importance of a time-dependent relationship between treatment with melatonin and mast cell stimulation became all the more vital for understanding the optimal conditions for inhibition by melatonin. HMC-1 cells were incubated with a 100  $\mu$ M concentration of melatonin for various time intervals (5 minutes, 10 minutes, 15 minutes, 20 minutes, and 30 minutes). The cells were then stimulated with mastoparan. The results showed a gradual decrease in the amount of  $\beta$ -hexosaminidase released over a time period of approximately 20 minutes. After this point,  $\beta$ -hexosaminidase release started increasing, suggesting that a 20-minute incubation time was optimal for melatonin's inhibitory effect. The decrease in melatonin's inhibitory effect may be due to possible mast cell sensitization to melatonin or to some cell-induced degradation of the hormone in the cellular microenvironment. Once again, in HMC-1 cells stimulated by neurotensin, this relationship did not exist, showing a

large range of variation and no consistent relationship between incubation time and melatonin-induced inhibition.

While  $\beta$ -hexosaminidase release is just one indicator of mast cell activation, another means of measuring mast cell activation is the release of stored histamine. Thus the release of histamine was also measured to corroborate the existing data or provide alternative explanations for melatonin and mast cell interactions. Additionally, it was decided that using a different model of mast cell activation might also prove useful in understanding this interplay. Mast cells were isolated from the pleural and peritoneal cavities of rats and incubated with 100  $\mu$ M melatonin for 15 minutes before stimulation with various concentrations of either mastoparan or neurotensin for another 15 minutes.

The resulting data was consistent with data gathered from the human HMC-1 and  $\beta$ -hexosaminidase model. While incubation with melatonin had no significant effect on mast cells activated by neurotensin, a trend did emerge in rat mast cells activated by mastoparan, especially at lower concentrations of the activator. At a mastoparan concentration of 10  $\mu$ M there was a 13.40% decrease in histamine release in cells incubated with melatonin (compared to cells incubated with the same concentration of mastoparan without melatonin). Even at a higher concentration of activator (20  $\mu$ M mastoparan) incubation with melatonin caused a ~3% decrease in histamine release in comparison to its matched negative control. Because these results are similar to the data gathered in experiments using human HMC-1 cells, this may imply that the mechanism for melatonin-induced inhibition of the mastoparan activated pathway could be very similar, if not identical, in the HMC-1 human mast cell and the rat mast cell.

The differential inhibition between mast cells stimulated with mastoparan and those stimulated with neurotensin may be attributed to the different receptor types. Mastoparan binds

via the Mas-gene related receptor isotype MrgX2, which binds basic molecules and activates G-proteins (29). Neurotensin receptors, on the other hand, bind specifically to neurotensin (27). While both ligands have been shown to trigger mast cell activation and degranulation, the fact that they are differentially regulated by melatonin suggests that these activation pathways possibly do not activate the same set of intracellular messengers. There is the possibility of both activation pathways converging at a single point along the pathway, but if so, the inhibitory event triggered by melatonin binding via the MT1 or MT2 receptor must happen further upstream so as to inhibit the mastoparan pathway and not the neurotensin pathway.

While this study certainly elucidated some important information regarding the interaction between mast cells and melatonin, much more research in this field is needed to improve the understanding of this interplay, but also the activity of mast cells in general. As there is some older evidence to suggest that mastoparan can travel through the cell membrane and act endogenously (30), research could be done to identify the intracellular pathways activated by the mastoparan receptor, potentially explaining the difference between the mastoparan and neurotensin pathways. Similarly, Western Blotting or immunocytochemistry should be performed on human HMC-1 cells to confirm the actual presence of the MT1 and MT2 melatonin receptors on the cell membrane in order to corroborate the mRNA expression data revealed in this study.

These ideas could have important implications in the way that melatonin treatment is administered, allowing potentially greater control mast cell-induced inflammatory events. Even with this data, however, there are many hurdles for melatonin and mast cell therapy in clinical settings. No definitive guidelines have been formulated for clinical evaluation of patients with low melatonin levels, primarily because a “melatonin deficiency syndrome” has not yet been deemed as an independent entity (16). Because of this, there are limited opportunities for

studying melatonin therapy. In addition, while perhaps most available, the murine and tumorigenic HMC-1 human mast cell lines are not perfect models and do not accurately represent a fully mature, active, human mast cell (3). Better models, such as the human LAD-2 mast cell lineage, that more accurately resemble mature mast cells do exist, but are often very expensive and difficult to maintain (25), limiting the research performed using these models. Also in terms of clinical usefulness, because all cancers are not the same, research has shown that both mast cells and melatonin interact differently with different types of cancers (9), making it harder to determine how effective a treatment will be given a specific type of cancer.

It appears that mast cells are a “necessary evil” we cannot live without (2), having the potential to both benefit and damage the human body. The results from this study only begin to explore the possible inhibition of human mast cells by melatonin. Information from studies in this field may have an impact on increasing the efficacy and/or efficiency of melatonin therapy. Melatonin therapy is just one of many mast cell-related potential treatments for not only cancer, but a plethora of diseases, both short-term and chronic, that have inflammatory components. Because of this, the continuation of mast cell research using human mast cell models is vital for expanding our understanding of mast cells and their activity, thereby continuing the close the information gap that currently exists.

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