Spring 5-1-2019

The Signaling Pathways of Metallothionein-Mediated Chemotaxis in Breast Cancer

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The Signaling Pathways of Metallothionein-Mediated Chemotaxis in Breast Cancer

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A University Scholar Project and Honors Thesis

May 2019

University of Connecticut

Department of Molecular and Cell Biology

Approvals:

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Abstract

Metallothionein (MT) is a small, thiol rich protein released into the extracellular environment in response to stress. Elevated expression of MT has been linked to many inflammatory diseases including inflammatory bowel diseases, diabetes, and cancer. In breast cancer, high expression of MT has been associated with poor patient prognosis. Previous studies have shown that MT acts as a chemoattractant in lymphocytes, and that UC1MT, a monoclonal anti-MT antibody, can block this chemotactic response. In addition, it has been shown that both Cholera toxin and Pertussis toxin, which are known antagonists of G-protein coupled receptors, can inhibit MT-mediated chemotaxis. Here, I investigate the signaling pathways of MT-mediated chemotaxis using small molecule inhibitors in cellular models of inflammation and breast cancer. I also examine the chemotactic potential of MT in mammary tumor cell lines and the ability of UC1MT to block this chemotactic response. Experiments revealed that MT is chemotactic in mammary tumor cell lines. I have found that, like in lymphocytes, MT interacts with receptor CXCR4 in breast cancer models to initiate the chemotactic response. Studies conducted using MT peptides revealed that MT’s N-terminus is likely involved in the binding of this protein to its receptor. MT-mediated chemotaxis in both immune cells and breast cancer cell models can be blocked using CK-666 and U73122, but not with PD98059, which suggests that the Arp2/3 complex and phospholipase C (PLC) are involved in MT’s chemotactic signaling pathways, but that mitogen activated kinase kinase (MEK) is not. Finally, I found that UC1MT can block MT-mediated chemotaxis in mammary tumor cell lines. This suggests that UC1MT may be a useful therapeutic to inhibit breast cancer metastasis.
Acknowledgements

First, I would like to thank all my lab members for their support and advice over the past few years, with a special thank you to Clare Melchiorre and former lab member Dr. Sadikshya Bhandari. I would also like to thank my PI, Dr. Michael Lynes, for his valuable mentorship and guidance throughout my first research experience. I thank my other committee members, Dr. Adam Zweifach and Dr. Nicole Broderick for their support of my University Scholar Project over the past year and a half. Finally, I would like to thank my friends and family, especially my parents and Charlie, for all your love and support. This University Scholar and Honors Project is partially funded by a Summer Undergraduate Research Fund (SURF) Award.
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Introduction

Metallothionein: Background and Significance

Metallothionein (MT), is a low molecular weight, thiol-rich protein, that is released by cells into the extracellular environment as a response to stress\(^1\). MT is highly conserved across many species, and in mammals there are four isoforms of the protein. MT-1 and MT-2 are expressed at low levels in almost all cell types throughout an organism, while MT-3 and MT-4 have more tissue specific expression\(^2\). The experiments conducted for this study focused on the MT-1 isoform, which will be referred to as MT from this point forward. Traditionally, MT has been studied in the intracellular environment and has been shown to play a role in metal homeostasis by interacting with divalent metal cations such as zinc, copper, cadmium, and mercury via the thiols present in its structure\(^3\). MT forms two metal binding domains, the C-terminal \(\alpha\)-domain and the N-terminal \(\beta\)-domain (Figure 1). The \(\alpha\)-domain is comprised of amino acids 31-61 and is capable of binding four divalent metals. The \(\beta\)-domain is comprised of amino acids 1-30 and can bind three divalent metals\(^4,5\).

![Figure 1. Mammalian Metallothionein Structure with Metal Binding Pockets\(^6\)](image-url)
Even though MT is typically studied in the intracellular environment, it also plays a significant role in the extracellular environment. It has been shown that MT has many immunomodulatory effects on lymphocytes. MT plays a role in CD4+ T cell differentiation, and CD8+ cytotoxic T cell and dendritic cell function. It has also been shown that MT exposure synergistically enhances T and B cell proliferation when used in conjunction with Concanavalin A and Lipopolysaccharide respectively.

Chemotaxis is defined as directional movement of cells or organisms in response to a chemical or other stimulus. The Lynes Lab has shown that immune cells migrate chemotactically towards increasing concentrations of MT and that MT exposure results in an increase in F-actin levels in cells. The addition of UC1MT, an anti-MT monoclonal antibody, alters the immunomodulatory effect of MT. In regard to this study, it is of particular interest that UC1MT can block MT’s chemotactic effect in Jurkat T cells.

G-protein coupled receptors (GPCRs) and their downstream signaling pathways are commonly involved in the initiation of a chemotactic response. A previous study done in the Lynes lab suggests that a GPCR may be involved in signaling associated with MT-mediated chemotaxis since the chemotactic response to MT is blocked by the presence of either pertussis toxin or cholera toxin, both of which are known antagonists of GPCR mediated events. These findings prompted my interest in investigating the signaling pathways of MT-mediated chemotaxis and the potential role of GPCRs in initiating these pathways.
Metallothionein and Inflammatory Diseases

MT has been linked to the progression of inflammation associated with disease in many organ systems. Cancer, neuroinflammatory diseases, inflammatory bowel disease, and diabetes have all been linked to elevated MT expression$^{15,16}$.

MT has been found to be upregulated in many neurodegenerative diseases including Alzheimer’s Disease, amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS). In addition, acute and chronic brain injury has been linked to increased levels of MT due to neuroinflammation and oxidative stress associated with these conditions$^{15,17}$.

Inflammatory Bowel Diseases (IBD) such as Crohn’s Disease and ulcerative colitis are chronic intestinal inflammatory diseases with unclear origins, however it is thought that genetic factors, environmental factors, and the gut microbiota contribute to the disease phenotype$^{18,19}$.
Studies have shown that MT expression levels are dysregulated in patients with IBD\textsuperscript{20}. Similarly, murine colitis models show upregulation of MTs\textsuperscript{21}. Experiments conducted in murine Dextran Sodium Sulphate (DSS)-induced colitis model suggest that UC1MT may be a useful tool as a preventative and therapeutic treatment for colitis as it reduces cell infiltration of the intestinal epithelium, which is characteristic of inflammation associated with colitis\textsuperscript{22}.

Type 1 Diabetes (T1D) is an autoimmune disease characterized by a loss of tolerance to insulin-producing pancreatic β cells, in which the immune system targets these cells for destruction. In T1D, pancreatic β cells are completely lost, which results in insulin deficiency and dysregulation of glucose metabolism\textsuperscript{23}. In a study examining the expression of MT at different stages of T1D development, it was shown that MT-1 and MT-3 levels were increased in patients with longstanding T1D compared to patients with early onset of the disease and control patients\textsuperscript{24}. Experiments conducted in non-obese diabetic mice, a mouse model commonly used for T1D research, have shown that administration of UC1MT results in a decreased blood glucose level and decreased insulitis score compared to administration of an isotype control\textsuperscript{25}, indicating that UC1MT may have a useful clinical application in the prevention of this disease.

**Metallothionein and Cancer**

Increased expression of MT has been linked to the manifestation of many different cancers, including glioblastoma, prostate cancer, ovarian, and breast cancer\textsuperscript{26–29}. The increased expression of MT in each of these types of cancer indicate a worse prognosis for the patient\textsuperscript{27,30}. MT can inhibit apoptosis and protect cells against radiation and cytotoxic alkylating agents. Because many chemotherapy agents fall into this category, MT can protect cells, including cancer cells, from the drugs used to eradicate them\textsuperscript{27}. This association between increased MT...
expression and decreased chemotherapy efficacy highlights a gap in current cancer treatment protocols, one that can potentially be filled by manipulation of the extracellular pool of MT with UC1MT.

**Metallothionein and Breast Cancer**

Breast Cancer represents a significant health risks, with an estimated 266,120 new cases and accounting for an estimated 40,920 deaths in the United States in 2018\(^\text{31}\). With one in eight women developing the disease in her lifetime, breast cancer is the most common cancer diagnosed and second leading cause of death in women. While 62\% of breast cancer cases involve only a primary tumor, 31\% of cases involved metastases to local lymph nodes, and 6\% of cases involve metastases to distant locations in the body. Once the tumor has extended beyond its primary location, five-year survival rates for patients drop significantly\(^\text{31}\). Even with modern therapeutics, metastasis still poses a significant challenge when treating patients with breast cancer and serves as an important target for new drug development.

Several studies have shown that inflammation plays a key role in the progression of breast cancer\(^\text{32–35}\). Of these studies, the one conducted by Pierce is of particular interest as it suggests that circulating markers of inflammation, including pro-inflammatory cytokines, might indicate disease recurrence even without a history of metastasis or the presence of other clinical indications of cancer\(^\text{32}\).

In breast cancer, increased expression of MT has been associated with greater tumor grade\(^\text{30}\). In addition, high levels of MT in breast cancer have been linked to worse tumor prognosis, including increased likelihood of local tumor recurrence, shorter disease-free interval,
and decreased survival\textsuperscript{36}. MT’s role in inflammation and its association with poor prognosis in breast cancer patients suggests that manipulation of this stress response protein may improve patient outcomes.

In this study, I will investigate the mechanism by which MT initiates a chemotactic response in immune cells and thus contributes to the inflammatory environment associated with many disease phenotypes. I will also examine this phenomenon in mammary tumor cell models to determine if using UC1MT to manipulate the extracellular pool of MT would be a useful therapeutic approach to inhibit breast cancer metastasis.
Materials and Methods

Proteins, Antibodies, and Small Molecule Inhibitors

MT-1 from rabbit liver (Cat# ALX-202-072-0000), CK-666 (Cat# ALX-270-506-M002), U73122 (Cat# BML-ST391-0005), and PD98059 (Cat# BML-EI360-0005) were obtained from Enzo Life Sciences. Recombinant human SDF-1α was obtained from Shenandoah (Cat# 100-20). AMD3100 (Cat# A5602) was obtained from Sigma Life Sciences. Thirteen different MT peptides [MT_{1-10} (Cat# 65595-1), MT_{4-12} (Cat# 65595-2), MT_{11-20} (Cat# 65595-3), MT_{16-25} (Cat# 65595-4), MT_{22-31} (Cat# 58274), MT_{25-35} (Cat# 65595-5), MT_{31-40} (Cat# 65595-6), MT_{35-43} (Cat# 65595-7), MT_{40-49} (Cat# 65595-8), MT_{46-55} (Cat# 65595-9), MT_{51-61} (Cat# 65595-10), MT_{sc1} (Cat# 58242-1), and MT_{sc2} (Cat# 65595-11)] were designed based on the rabbit MT-1 sequence (Table 1) as described in Bhandari, 2018 and purchased from AnaSpec. UC1MT, a monoclonal anti-MT antibody, was obtained from Thermo Fisher Scientific.

<table>
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<td>MT_{sc2}</td>
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</tr>
</tbody>
</table>

Table 1. Rabbit MT-1 and MT Peptide Sequences
Cell Culture

Jurkat T Cells (ATCC TIB-152), a human T lymphocyte leukemia cell line, were cultured in RPMI-1640 (Sigma, Cat# 8758) with 1% penicillin/streptomycin (Cellgro, Cat# 30-002-CI), 1mM Sodium Pyruvate (MP Biomedicals, Cat# 1682049), 1mM HEPES Buffer (Corning, Cat# 25-060-CI), 4mM L-Glutamine (Sigma, Cat# 56-58-9), and 10% heat inactivated Fetal Bovine Serum (Atlanta Biologicals, Cat# S11550) at 37°C with 5% CO₂. Cells were maintained at a concentration of between 10⁵ and 10⁶ cells per milliliter by aspirating cells in spent media and replenishing with fresh media using sterile technique.

MDA-MB-231 (ATCC HTB-26), a triple negative human epithelial breast cancer cell line, was cultured in high glucose Dulbecco's Modified Eagle Medium (Gibco Life Sciences, Cat# 11965118) with 1% penicillin/streptomycin, 4mM L-Glutamine, and 10% heat inactivated Fetal Bovine Serum at 37°C with 5% CO₂. Cells were maintained at a concentration of between 10⁵ and 10⁶ cells per milliliter by aspirating cells in spent media and replenishing with fresh media using sterile technique. Because MDA-MB-231 cells are adherent, a cell scraper (Fisherbrand, Cat# 08-100-241) was used to dislodge them from the bottom of the flask before each passage.

419, a murine mammary tumor cell line derived from the MMTV-PyMT mouse model, was obtained from David Lawrence, PhD (New York State Department of Health Wadsworth Center, Albany NY) and cultured in high glucose Dulbecco's Modified Eagle Medium with 1% penicillin/streptomycin and 10% heat inactivated Fetal Bovine Serum at 37°C with 5% CO₂. Cells were maintained at a concentration of between 10⁵ and 10⁶ cells per milliliter by aspirating cells in spent media and replenishing with fresh media using sterile technique. Because 419 Cells
are a highly adherent cell line, they were treated with Trypsin-EDTA (Sigma Life Sciences, Cat# T2601) for five minutes then dislodged using a cell scraper before each passage.

**Primary Splenocyte Isolation**

C57BL/6J mice were euthanized by CO₂ asphyxiation. The spleen was harvested using sterile dissection technique. A single cell suspension was made using a disposable pestle tissue grinder. Cell suspension was put through a 40µm pore cell strainer (Fisherbrand, Cat# 22363547) and washed with 4mL of splenocyte media (RPMI-1640 with 1% penicillin/streptomycin, 1mM sodium pyruvate, 1mM L-Glutamine, 0.1 mM non-essential amino acids (Gibco, Cat# 01216), and 10% heat inactivated Fetal Bovine Serum). Cells were washed with 10mL of splenocyte media then the red blood cells were lysed using sterile Ammonium-Chloride-Potassium Lysis Buffer (150mM ammonium chloride, 10mM potassium bicarbonate, and 0.1mM Na₂EDTA at pH 7.2). Cells were washed with 10mL splenocyte media. Splenocytes were resuspended in 10mL fresh media and put through a 40µm pore cell strainer. Cell density and viability were calculated using a hemocytometer and trypan blue (Sigma Life Sciences, Cat# T8154) exclusion staining.

**Boyden Chamber Assay**

The Boyden Chamber Assay was conducted in either the 48 (Neuro Probe, Cat# AP48) or 96 (Neuro Probe, Cat# AP96) well Micro Chemotaxis Chamber. Chemoattractants were diluted to working concentrations in appropriate media. 25µL of chemoattractant were added to the bottom wells of the Boyden Chamber. Media alone served as a negative control. Incubation
times and membrane pore size were optimized for each cell line used. For Jurkat T cells, experiments were conducted in the 48 well chamber using 5µm pore membranes (Neuro Probe, Cat# PFB5). For primary mouse splenocytes, experiments were conducted in the 48 well chamber using 3µm pore membranes (Neuro Probe, Cat# PFB3). For MDA-MB-231 cells, experiments were conducted in the 48 well chamber using 8µm pore membranes (Neuro Probe, Cat# PFB8) coated with 10µg/mL Fibronectin (Sigma Life Sciences, Cat# F0895) in phosphate buffered saline (PBS) overnight prior to their use. For 419 cells, MT peptide experiments were conducted in the 96 well chamber, while all other experiments were conducted in the 48 well chamber. 3µm pore membranes (Neuro Probe, Cat# PFB3 or PFD3) were used for experiments using 419 cells. After chamber assembly, 50µl of cells in single cell suspension at 2x10^6 cells/mL were added to the wells of the upper chamber. For experiments involving small molecule inhibitors, cells were pre-incubated with non-toxic doses of either AMD3100 (Figure S1), CK-666 (Figure S2), U73122 (Figure S3), or PD98059 (Figure S4). After pre-incubation, cells were washed and resuspended at 2 million cells/mL to be loaded into the wells of the upper chamber. Chambers were incubated at 37°C with 5% CO₂ for 3 hours (Jurkat T cells, primary mouse splenocytes, and 419 cells) or 6 hours (MDA-MB-231 cells). After incubation, chambers were disassembled, and a cell scraper was used to remove the cells from the top side of the membrane. Cells on the underside of the membrane were stained using the Hema 3 stain set (Protocol, Cat# 122-911) and were counted under the light microscope at 10x.

Data Analysis

Data was analyzed using the GraphPad Prism 8 software. Each experiment was analyzed using ordinary one-way analysis of variance (ANOVA) followed by Tukey’s Multiple
Comparisons Test to determine statistical significance. For each statistical test, ns (no significance) indicates a p value of greater than 0.05, * indicates a p value of less than or equal to 0.05, ** indicates a p value of less than or equal to 0.01, *** indicates a p value of less than or equal to 0.001, and **** indicates a p value of less than or equal to 0.0001. There was a minimum of three replicates for each condition in every experiment and the average of these values was calculated to be used for analysis. All values are displayed with standard deviation. Graphs are representative results of three independent experiments.
Results

MT is Chemotactic in Mammary Tumor Cell Lines

Previous research conducted in the Lynes Lab has established that MT acts as a chemoattractant to lymphocytes\textsuperscript{11,25}. As a result, immune cells migrate towards increasing concentrations of MT in the body, which contributes to inflammation at sites of high MT expression. This phenomenon contributes to many inflammatory diseases including inflammatory bowel disease, diabetes, and many types of cancer\textsuperscript{15}. As mentioned earlier, MT expression is associated with worse outcomes in cancer patients, including those with breast cancer\textsuperscript{27,30}. MT expression contributes to inflammation found in the tumor micro environment which plays a key role in the development of the disease\textsuperscript{32–35}. While the chemotactic role of MT in the extracellular environment has been studied extensively in Bhandari, 2018, these studies did not address the role of MT-mediated chemotaxis in breast cancer cells.

For these experiments, I have used two mammary tumor cell lines as models for breast cancer. MDA-MB-231 is a triple negative human epithelial breast cancer cell line commonly used in breast cancer research. I selected this cell line to model breast cancer in this project as triple negative breast cancer is particularly challenging to treat with the current available therapeutics, so it is important to continue to develop new options for treating this disease phenotype. 419, a murine mammary tumor cell line derived from MMTV-PyMT mice served as a second model of mammary tumorigenesis. I have selected this murine cell line to model mammary tumorigenesis as mice are commonly used animal models in breast cancer research, and the MMTV-PyMT mouse model, from which this cell line is derived from, develops particularly aggressive mammary tumors, which are challenging to treat.
In Figure 3, I have conducted experiments to determine if MT is chemotactic in breast cancer. If MT can induce chemotaxis in these mammary tumor cell lines, this may suggest that MT plays a role in breast cancer metastasis, thereby resulting in a worse prognosis for the patient.

Using the Boyden Chamber assay, I was able to examine the effect of MT on both MDA-MB-231 cells and 419 cells. For these experiments, either media alone or MT was loaded into the bottom wells of the chamber. The chamber was then assembled and MDA-MB-231 cells (Figure 3A) or 419 cells (Figure 3B) were loaded with and without MT into the wells of the upper chamber. When MT was loaded into the wells of the bottom chamber, there was a statistically significant increase in the average number of cells migrated per well in both MDA-MB-231 cells and 419 cells, however when MT was loaded into the wells of the upper chamber with the cells, there was no increase in cell migration. These results indicated that MT is in fact chemotactic in MDA-MB-231 cells and 419 cells. Because MT is chemotactic in these models of breast cancer, it suggests that MT may play a role in breast cancer metastasis.
Figure 3. MT is Chemotactic in Mammary Tumor Cell Lines.

The bottom chamber was loaded with either 10µM MT or media alone. Boyden chamber was assembled using an 8µm pore (A) or 3µm pore (B) membrane. 8µm pore membranes were coated in 10µg/mL fibronectin at 4°C overnight prior to use. MDA-MB-231 (A) or 419 (B) cells (2million cells/mL) were loaded into the top chamber with either 10µM MT or media alone. Boyden Chamber was incubated for 6 hours (A) or 3 hours (B) at 37°C in 5% CO₂. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001. Graphs are representative of three independent experiments.
MT Peptides and the Chemotactic Response in Mammary Tumor Cell Lines

Previous studies in the Lynes Lab have shown that pre-incubation with MT can block SDF-1α-mediated chemotaxis in Jurkat T cells. This finding prompted the development of MT peptides using the sequence of MT-1 (Table 1) to determine which region of MT binds to its receptor(s) to initiate the chemotactic response. Experiments conducted using Jurkat T cells indicated that the N-terminal region of MT interacts with its receptor25. In the experiments conducted in Figure 4, I used MT peptides to characterize MT’s interaction with its receptor in breast cancer using 419 cells as a model of mammary tumorigenesis.

In Figure 4A, I used the Boyden Chamber assay to determine if MT peptides are chemotactic in 419 cells. If any of the MT peptides are chemotactic, this would indicate that those amino acids are responsible for interacting with MT’s receptor(s) on the cell surface to initiate the chemotactic response. MT peptides, MT (MT control), or media alone (media control) were loaded into the wells of the bottom chamber. Boyden Chamber was assembled, and 419 cells were loaded into the wells of the upper chamber. Results from this experiment showed that none of the MT peptides caused an increase in cell migration compared to the Media control, which suggests that they are not chemotactic on their own. While these results did not provide any information as to which amino acids interact with MT’s receptor, they do suggest that there is some important interaction between full-length MT and the receptor that shortened peptides alone cannot establish.

In Figure 4B, I investigated the ability of MT peptides to block MT-mediated chemotaxis in 419 cells. If any MT peptide occupies the binding site for MT on the receptor, it will inhibit MT binding, thus blocking MT’s chemotactic effect. 419 cells were pre-incubated with MT peptides, MT or media alone (MT control and Media control). The wells of the bottom chamber
were loaded with MT (MT peptides, MT, and MT Control) or media alone (Media control). The Boyden Chamber was assembled, and the wells of the top chamber were loaded with 419 cells. I found that pre-incubation with MT\textsubscript{1-10}, MT\textsubscript{4-12}, and MT\textsubscript{11-20} results in a reduction in cell migration compared to the MT control, suggesting that these peptides can block MT-mediated chemotaxis.

In Figure 4C, I examined the ability of MT peptides to block Stromal Cell-Derived Factor-1\textalpha (SDF-1\textalpha)-mediated chemotaxis in 419 cells. The design of this experiment was similar to that of the experiment conducted in Figure 4B, however SDF-1\textalpha was used as the chemoattractant instead of MT. Since it has been shown that MT can block SDF-1\textalpha-mediated chemotaxis\textsuperscript{25}, the ability of one (or more) of the MT peptides to block this response would indicate that that region of MT is responsible for binding to MT’s receptor(s). 419 cells were pre-incubated with MT peptides, MT or media alone (SDF-1\textalpha control and Media control). The wells of the bottom chamber were loaded with SDF-1\textalpha (MT peptides, MT, and SDF-1\textalpha Control) or media alone (Media control). The Boyden Chamber was assembled, and the wells of the top chamber were loaded with 419 cells. I found that pre-incubation with MT\textsubscript{1-10}, MT\textsubscript{4-12}, and MT\textsubscript{11-20} results in a reduction in cell migration compared to the SDF-1\textalpha control, suggesting that these peptides can block SDF-1\textalpha-mediated chemotaxis. These results are consistent with those found in experiments using MT as the chemoattractant (Figure 4B).

While the MT peptides were not able to elicit a chemotactic response on their own, a few of peptides corresponding with the N-terminus of the protein (MT\textsubscript{1-10}, MT\textsubscript{4-12}, and MT\textsubscript{11-20}) were able to block both MT-mediated and SDF-1\textalpha-mediated chemotaxis in 419 cells. Taken together, the results from experiments conducted in Figure 4 suggest that there is some important
interaction between full-length MT and its receptor that is necessary to illicit the chemotactic response that MT peptides alone cannot establish, and that the N-terminus of MT is responsible for binding to its receptor.
Figure 4. MT Peptides and the Chemotactic Response in Mammary Tumor Cell Lines

(A) Chemoattractants (100μg/mL MT peptides, 100μg/mL MT or media alone) were loaded into the bottom chamber. Boyden chamber was assembled using a 3μm pore membrane. 419 cells (2million cells/mL) were loaded into the upper chamber. (B) 419 cells were pre-incubated for 1 hour with either 100μg/mL MT peptides, 100μg/mL MT or media alone (MT control and Media control) at 37°C in 5% CO₂. 10μM MT (MT peptides, MT, and MT control) or media alone (Media control) was loaded into the bottom chamber. Boyden chamber was assembled using a
3µm pore membrane. Cells were washed, resuspended at 2million cells/mL, and loaded into the upper chamber. (C) 419 cells were pre-incubated for 1 hour with either 100µg/mL MT peptides, 100µg/mL MT or media alone (SDF-1α control and Media control) at 37°C in 5% CO₂. 100ng/mL SDF-1α (MT peptides, MT, and SDF-1α control) or media alone (Media control) was loaded into the bottom chamber. Boyden chamber was assembled using a 3µm pore membrane. Cells were washed, resuspended at 2million cells/mL, and loaded into the upper chamber. (A+B+C) Boyden chamber was incubated for 3 hours at 37°C in 5% CO₂. Cells on the underside of the membrane were enumerated using a light microscope under 10x. Experimental conditions were compared to the Media control (A), the MT control (B) or the SDF-1α control (C) and One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001. Graphs are representative of three independent experiments.

**MT Interacts with CXCR4 to Initiate the Chemotactic Response in Mammary Tumor Cell Lines**

Previous studies in the Lynes Lab have shown that MT can interfere with SDF-1α-CXCR4 mediated chemotaxis. In addition, it has been shown that MT-mediated chemotaxis can be blocked by Cholera toxin and Pertussis toxin, both of which are known antagonists of GPCRs. These findings suggested that MT interacts with CXCR4 to initiate the chemotactic response.

AMD3100 (AnorMeD 3100), originally known as JM3100, is a small molecule inhibitor of CXCR4 that was initially identified for its ability to target HIV viral protein gp120. Later
studies found that AMD3100 blocked the interaction between gp120 and chemokine receptor CXCR4, preventing the entry of HIV virus into the cell\textsuperscript{37,38}. Currently, AMD3100 is being examined for its ability to act as an HIV entry inhibitor, to mobilize stem cells, and to treat leukemias and solid tumors\textsuperscript{39}. 

AMD3100 has been shown to block MT-mediated chemotaxis in Jurkat T cells, which suggests that CXCR4 plays a role in MT’s chemotactic response in lymphocytes and thus inflammation associated with MT expression\textsuperscript{25}. In these experiments, I will determine if AMD3100 also blocks MT-mediated chemotaxis in mammary tumor cell models. If AMD3100 blocks MT-mediated chemotaxis in MDA-MB-231 cells and 419 cells, this would suggest that CXCR4 is involved in MT’s chemotactic signaling pathways in breast cancer.

In Figure 5, MDA-MB-231 cells (Figure 5A) or 419 cells (Figure 5B) were pre-incubated with non-toxic doses of AMD3100 (Figure S1). MT control and Media control cells were pre-incubated in media alone. MT (MT control and AMD3100) or media alone (Media control) was loaded into the wells of the bottom chamber. The Boyden Chamber was assembled, and cells were loaded into the wells of the upper chamber. Pre-incubation with AMD3100 results in a statistically significant decrease in the average number of cells migrated per well in both MDA-MB-231 cells (Figure 5A) and 419 cells (Figure 5B).

The fact that pre-incubation with AMD3100 blocks MT-mediated chemotaxis in mammary tumor cell models suggests that CXCR4 is involved in MT’s chemotactic signaling pathways. While CXCR4 may not be the only receptor that MT interacts with to initiate its chemotactic response, these results provide valuable insight into MT’s mechanism of action as a chemoattractant, which will help us understand how manipulating the extracellular pool of MT may have therapeutic benefits in breast cancer.
Figure 5. CXCR4 is Involved in MT-Mediated Chemotaxis in Mammary Tumor Cell Lines.

Cells were pre-incubated with AMD3100 for 30 minutes at 37°C in 5% CO₂. 20µg/mL AMD3100 was used with MDA-MB-231 cells (A), while 10µg/mL AMD3100 was used with 419 cells (B) as determined by dose response and viability experiments (Fig. S1). Media and MT controls were pre-incubated under the same conditions in media alone. The bottom chamber was loaded with either 10µM MT (MT control and AMD3100) or media alone (Media control). Boyden chamber was assembled using an 8µm (A) or 3µm pore (B) membrane. 8µm pore membranes were coated in 10µg/mL fibronectin at 4°C overnight prior to use. Cells were washed in media, resuspended at 2 million cells/mL, and loaded into the top chamber. Boyden Chamber was incubated for 6 hours (A) or 3 hours (B) at 37°C in 5% CO₂. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05.
0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001. Graphs are representative of three independent experiments.

The Arp2/3 Complex is Involved in MT-Mediated Chemotaxis

Actin Related Protein 2/3 (Arp2/3) complex plays an important role in the regulation of the actin cytoskeleton by catalyzing actin filament nucleation. Arp2/3 bound to existing actin filaments interacts with ATP and nucleation-promoting factors to facilitate the addition of actin monomers to the filament\textsuperscript{40,41}. As previously discussed, MT exposure results in an increase in F-actin concentrations in cells\textsuperscript{11}. This led to the hypothesis that the Arp2/3 complex is involved in MT’s chemotactic signaling pathways.

CK-666, a small molecule inhibitor of the Arp2/3 complex, was used to test this hypothesis. CK-666 inhibits the activity of the Arp2/3 complex by stabilizing the inactive state of the complex. CK-666 blocks the movement of both the Arp2 and the Arp3 subunits into the active conformation, thereby preventing the nucleation of actin filament\textsuperscript{42}.

First, I examined the role of the Arp2/3 complex in MT-mediated chemotaxis as it relates to infiltration of immune cells and subsequent inflammation. For these experiments, I pre-incubated Jurkat T cells (Figure 6A) or primary mouse splenocytes (Figure 6B) with nontoxic doses of CK-666 (Figure S2). MT control and Media control cells were pre-incubated in media alone. MT (MT control and CK-666) or media alone (Media control) was loaded into the wells of the bottom chamber. After the Boyden Chamber was assembled, cells were loaded into the wells of the top chamber. Pre-incubation with CK-666 results in a statistically significant
decrease in the average number of cells migrated per well in both Jurkat T cells (Figure 6A) and primary mouse splenocytes (Figure 6B).

Next, I examined the role of the Arp2/3 complex in MT-mediated chemotaxis as it relates to breast cancer metastasis. For these experiments, I pre-incubated MDA-MB-231 cells (Figure 6C) or 419 cells (Figure 6D) with nontoxic doses of CK-666 (Figure S2). MT control and Media control cells were pre-incubated in media alone. MT (MT control and CK-666) or media alone (Media control) was loaded into the wells of the bottom chamber. After the Boyden Chamber was assembled, cells were loaded into the wells of the top chamber. Pre-incubation with CK-666 results in a statistically significant decrease in the average number of cells migrated per well in both MDA-MB-231 cells (Figure 6C) and 419 cells (Figure 6D).

The fact that pre-incubation with CK-666 blocks MT-mediated chemotaxis in lymphocytes and mammary tumor cell lines suggests that the Arp2/3 complex is involved in MT’s chemotactic signaling pathways. This result is significant as it provides valuable insight regarding the mechanism by which MT induces chemotaxis. By understanding MT’s mechanism of action, we can identify the therapeutic opportunities of manipulating the extracellular pool of MT to reduce inflammation and prevent breast cancer metastasis.

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Figure 6. Arp2/3 is Involved in MT-Mediated Chemotaxis.

Jurkat T cells (A), Primary Mouse Splenocytes (B), MDA-MB-231 cells (C) or 419 cells (D) were pre-incubated with 100µM CK-666 for 1 hour at 37°C in 5% CO$_2$ as determined by dose response and viability experiments (Fig. S2). Media and MT controls were pre-incubated under the same conditions in media alone. The bottom chamber was loaded with either 10µM MT (MT control and CK-666) or media alone (Media control). Boyden chamber was assembled using an 8µm (C), 5µM (A), or 3µm pore (B+D) membrane. 8µm pore membranes were coated in
10µg/mL fibronectin at 4°C overnight prior to use. Cells were washed in media, resuspended at 2 million cells/mL, and loaded into the top chamber. Boyden Chamber was incubated for 3 hours (A+B+D) or 6 hours (C) at 37°C in 5% CO₂. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001. Graphs are representative of three independent experiments.

**Phospholipase C is Involved in MT-Mediated Chemotaxis**

Phospholipase C (PLC) β has been shown to be involved in G-protein coupled receptor signaling pathways in T lymphocytes. GPCR subunit Gαi activates PLCβ and phosphoinositol-3-kinase (PI3K) γ, which results in modification of the phospholipids produced by phosphatidylinositol 4,5-bisphosphate (PIP₂). Subsequently, the hydrolysis of PIP₂ results in the production of 1,4,5-triphosphate (IP₃), which causes an increase in intracellular calcium. This PLCβ signaling cascade has been shown to be involved in the chemotactic response initiated by several different chemokines including SDF-1α⁴³.

To investigate the role of PLC in MT-mediated chemotaxis, I used small molecule inhibitor U73122. U73122 blocks PLC signaling by reducing the availability of PIP₂, a substrate of the PLC signaling cascade⁴⁴.

First, I conducted experiments using Jurkat T cells and primary mouse splenocytes to determine the involvement of PLC in MT-mediated chemotaxis in the infiltration of immune cells. Jurkat T cells (Figure 7A) and primary mouse splenocytes (Figure 7B) were pre-incubated
with non-toxic doses of U73122 (Figure S3). MT control and Media control cells were pre-
incubated in media alone. MT (MT control and U73122) or media alone (Media control) was 
loaded into the wells of the bottom chamber. After the Boyden Chamber was assembled, cells 
were loaded into the wells of the top chamber. Pre-incubation with U73122 results in a 
statistically significant decrease in the average number of cells migrated per well in both Jurkat T 
cells (Figure 7A) and primary mouse splenocytes (Figure 7B).

Next, I examined the role of PLC in MT-mediated chemotaxis as it relates to breast 
cancer metastasis. For these experiments, I pre-incubated MDA-MB-231 cells (Figure 7C) and 
419 cells (Figure 7D) with U73122 at non-toxic doses (Figure S3). MT control and Media 
control cells were pre-incubated in media alone. MT (MT control and U73122) or media alone 
(Media control) was loaded into the wells of the bottom chamber. Boyden chamber was 
assembled, and cells were loaded into the wells of the top chamber. Pre-incubation experiments 
showed that U73122 treatment results in a statistically significant decrease in the average 
number of cells migrated per well in both MDA-MB-231 cells (Figure 7C) and 419 cells (Figure 
7D).

Results from U73122 pre-incubation experiments indicate that U73122 can block MT-
mediated chemotaxis in both lymphocytes and mammary tumor cell lines, which suggests that 
PLC is involved in MT’s chemotactic signaling pathways. These experiments have provided 
valuable information regarding the mechanism by which MT elicits a chemotactic response. This 
will allow us to more accurately understand the therapeutic potential of manipulating the 
extracellular pool of MT in both inflammation and breast cancer.
Figure 7. PLC is Involved in MT-Mediated Chemotaxis.

Jurkat T cells (A), Primary Mouse Splenocytes (B), MDA-MB-231 cells (C) or 419 cells (D) were pre-incubated with 1µM (A+B) or 5µM (C+D) U73122 for 30 minutes in at 37°C in 5% CO₂ as determined by dose response and viability experiments (Fig. S3). Media and MT controls were pre-incubated under the same conditions in media alone. The bottom chamber was loaded with either 10µM MT (MT control and U73122) or media alone (Media control). Boyden chamber was assembled using an 8µm (C), 5µM (A), or 3µm pore (B+D) membrane. 8µm pore
membranes were coated in 10µg/mL fibronectin at 4°C overnight prior to use. Cells were washed in media, resuspended at 2 million cells/mL, and loaded into the top chamber. Boyden Chamber was incubated for 3 hours (A+B+D) or 6 hours (C) at 37°C in 5% CO₂. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001. Graphs are representative of three independent experiments.

**MEK is Not Involved in MT-Mediated Chemotaxis**

The mitogen activated protein kinase (MAPK) signaling pathway is involved in many cellular processes including development, differentiation, and proliferation. Small G proteins known as RAS can phosphorylate mitogen activated protein kinase kinase kinase (MAPKKK) to activate it. MAPKK then activates MAPKK (MEK). Following their activation, MEKs can then phosphorylate tyrosine and threonine residues on extracellular signaling regulated kinase (ERK) to activate it\(^45\).

Previous studies conducted in the Lynes Lab have shown that MT can stimulate ERK1/2 activation, but that this activation only lasts a short amount of time (about two minutes)\(^25\). These results led to my interest in investigating the role of MEK in MT-mediated chemotaxis. To examine MEK’s involvement in MT’s chemotactic signaling cascades, I used PD98059, a small molecule inhibitor of MEK. PD98059 binds to the inactive form of MEK to prevent its activation by upstream activators\(^46\).
First, I conducted experiments using Jurkat T cells and primary mouse splenocytes to determine the involvement of MEK in MT-mediated chemotaxis in the infiltration of immune cells. Jurkat T cells (Figure 8A) and primary mouse splenocytes (Figure 8B) were pre-incubated with non-toxic doses of PD98059 (Figure S4). MT control and Media control cells were pre-incubated in media alone. MT (MT control and PD98059) or media alone (Media control) was loaded into the wells of the bottom chamber. After the Boyden Chamber was assembled, cells were loaded into the wells of the top chamber. These results show that pre-incubation with PD98059 has no effect on the average number of cells migrated per well in both Jurkat T cells (Figure 8A) and primary mouse splenocytes (Figure 8B).

Next, I used mammary tumor cell lines to determine if MEK is involved in MT-mediated chemotaxis in breast cancer. MDA-MB-231 cells (Figure 8C) and 419 cells (Figure 8D) were pre-incubated with non-toxic doses of PD98059 (Figure S4). MT control and Media control cells were pre-incubated in media alone. MT (MT control and PD98059) or media alone (Media control) was loaded into the wells of the bottom chamber. After the Boyden Chamber was assembled, cells were loaded into the wells of the top chamber. These results show that pre-incubation with PD98059 has no effect on the average number of cells migrated per well in both MDA-MB-231 cells (Figure 8C) and primary mouse splenocytes (Figure 8D).

Results from PD98059 experiments show that pre-incubation with this small molecule inhibitor does not block MT-mediated chemotaxis in both immune cells and mammary tumor cells lines, which suggests that MEK is not involved in MT’s chemotactic signaling pathways.
Figure 8. MEK is Not Involved in MT-Mediated Chemotaxis.

Jurkat T cells (A), Primary Mouse Splenocytes (B), MDA-MB-231 cells (C) or 419 cells (D) were pre-incubated with 50µM, 20µM, or 1µM PD98059 for 1 hour at 37°C in 5% CO₂. PD98059 concentrations were determined to be non-toxic in viability experiments (Fig. S4). Media and MT controls were pre-incubated under the same conditions in media alone. The bottom chamber was loaded with either 10µM MT (MT control and PD98059) or media alone (Media control). Boyden chamber was assembled using an 8µm (C), 5µM (A), or 3µm pore
(B+D) membrane. 8µm pore membranes were coated in 10µg/mL fibronectin at 4°C overnight prior to use. Cells were washed in media, resuspended at 2 million cells/mL, and loaded into the top chamber. Boyden Chamber was incubated for 3 hours (A+B+D) or 6 hours (C) at 37°C in 5% CO₂. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001. Graphs are representative of three independent experiments.

**UC1MT Blocks MT-Mediated Chemotaxis in Breast Cancer Cell Models**

UC1MT, a monoclonal anti-MT antibody, has been shown to alter the immunomodulatory effects of MT in immune cells¹²,¹³. Previous studies in the Lynes lab have shown that UC1MT can block MT-mediated chemotaxis in both Jurkat T cells and primary mouse splenocytes¹¹,²⁵. These results suggested that UC1MT may have therapeutic benefits in inflammatory diseases. A subsequent study conducting in a DSS-induced mouse model of colitis showed that UC1MT can reduce immune cell infiltration and inflammation in the gut, indicating that it may be a useful therapeutic to treat inflammatory bowel diseases²². Additionally, a study conducted in female NOD mice, which serve as a murine model of Type 1 Diabetes showed that treatment with UC1MT can prevent the development of Type 1 Diabetes²⁵. While UC1MT has shown great promise in the treatment in inflammatory diseases, it has not yet been evaluated in breast cancer models. Here, I will examine the ability of UC1MT to block MT-mediated chemotaxis in mammary tumor cell lines.
For these experiments, I pre-incubated media, MT, and SDF-1α with UC1MT. Then, I loaded the wells of the bottom chamber with either media, MT, or SDF-1α with and without UC1MT pre-incubation. After assembly of the Boyden chamber, MDA-MB-231 cells (Figure 9A) or 419 cells (Figure 9B) were loaded into the wells of the upper chamber. These results show that MT pre-incubation with UC1MT results in a decrease in the average number of cells migrated per well in both MDA-MB-231 cells (Figure 9A) and 419 cells (Figure 9B), but that media or SDF-1α pre-incubation with MT does not result in this effect.

The reduction in cell migration seen when MT is pre-incubated with UC1MT suggests that UC1MT blocks MT-mediated chemotaxis in mammary tumor cell lines. The fact that SDF-1α pre-incubation with UC1MT does not result in a reduction in the chemotactic response suggests that UC1MT specifically binds to MT to block its chemotactic response. Because UC1MT can block MT-mediated chemotaxis in mammary tumor cell lines, it may serve as a useful therapeutic to inhibit breast cancer metastasis.
Figure 9. UC1MT Blocks MT-Mediated Chemotaxis in Breast Cancer Cell Models.

Chemoattractants were pre-incubated with 50µg/mL UC1MT or in media alone for 1 hour at room temperature and then loaded into the bottom chamber. Boyden chamber was assembled using an 8µm pore (A) or 3µm pore (B) membrane. 8µm pore membranes were coated in 10µg/mL fibronectin at 4°C overnight prior to use. MDA-MB-231 (A) or 419 (B) cells (2 million cells/mL) were loaded into the top chamber. Boyden Chamber was incubated for 6 hours (A) or 3 hours (B) at 37°C in 5% CO₂. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001. Graphs are representative of three independent experiments.
Discussion

MT expression has been linked to inflammation and many disease phenotypes including IBD, diabetes, and many types of cancer\textsuperscript{15,16,20}. Previous studies in the Lynes Lab have shown that MT acts as a chemoattractant in lymphocytes and that MT-mediated chemotaxis can be blocked by UC1MT, a monoclonal anti-MT antibody\textsuperscript{11,25}. It has also been shown that GPCR antagonists Cholera toxin and Pertussis toxin can block MT’s chemotactic effect\textsuperscript{11}, which suggests that MT may interact with a GPCR to initiate its chemotactic signaling cascades. Here, I have investigated the mechanism by which MT initiates a chemotactic response in both inflammation and breast cancer.

Breast cancer is the second leading cause of death in women in the United States, with one in eight women developing the disease in her lifetime\textsuperscript{31}. Elevated MT expression has been linked to inflammation and poor prognosis in patients with breast cancer\textsuperscript{32–36}. MT’s association with breast cancer led to the interest in examining the chemotactic potential of MT in breast cancer cell models. In these experiments, MDA-MB-231 cells (Figure 3A) and 419 cells (Figure 3B) served as cell models for breast cancer. Results from these experiments showed that MT acts as a chemoattractant in mammary tumor cell lines, which suggests that MT may play a role in breast cancer metastasis. This finding prompted exploration of the mechanism by which MT initiates the chemotactic response and if UC1MT would block MT-mediated chemotaxis in breast cancer cell models.

Next, MT peptides were used to determine which region of full-length MT interacts with a receptor on the surface of cells to initiate its chemotactic signaling pathways. Previous studies in the Lynes Lab examined the ability of MT peptides to block SDF-1\textalpha-mediated chemotaxis in Jurkat T cells. These studies suggested that the N-terminus of MT is responsible for MT’s
binding to its receptor(s)\textsuperscript{25}. While these results indicate that the N-terminus of MT binds to its receptor(s) in lymphocytes, it had not yet been explored if the same was true in mammary tumor cell lines. For these experiments, I used 419 cells as a model for mammary tumorigenesis. It was found that MT peptides are not chemotactic on their own, but that MT\textsubscript{1-10}, MT\textsubscript{4-12}, and MT\textsubscript{11-20} can block both MT and SDF-1\textalpha-mediated chemotaxis in breast cancer cell models. These results suggest that the N-terminus of MT is responsible for binding to its receptor(s) and are consistent with the results of experiments conducted in lymphocytes.

AMD3100, a small molecule inhibitor of receptor CXCR4, was used to determine the involvement of CXCR4 in MT’s chemotactic signaling in mammary tumor cell lines. Studies conducted in Jurkat T cells found that MT can block SDF-1\textalpha-CXCR4 mediated chemotaxis. Subsequently, AMD3100 was used to determine the involvement of CXCR4 in MT-mediated chemotaxis in lymphocytes. It was found that AMD3100 can block MT-mediated chemotaxis in lymphocytes, which suggests that MT interacts with CXCR4 to initiate its chemotactic response\textsuperscript{25}. Similarly, experiments conducted in mammary tumor cell lines show that AMD3100 blocks MT-mediated chemotaxis (Figure 5). This suggests that CXCR4 is involved in the initiation of MT’s signaling cascades in both lymphocytes and breast cancer cell models.

MT exposure has been shown to increase F-actin levels in lymphocytes\textsuperscript{11}, which suggests the involvement of the Arp2/3 complex the signaling pathways of MT-mediated chemotaxis. Small molecule inhibitor CK-666 was used to evaluate the involvement of the Arp2/3 complex in MT-mediated chemotaxis in both lymphocytes and mammary tumor cell lines. CK-666 pre-incubation blocks MT-mediated chemotaxis immune cells (Figure 6 A+B) and mammary tumor cells (Figure 6 C+D), which suggests that the Arp2/3 complex is involved in MT’s chemotactic signaling pathways.
PLCβ signaling results in an increase in intracellular calcium levels and is known to be involved in SDF-1α-CXCR4 mediated chemotaxis\(^{43}\). U73122, a small molecule inhibitor of PLC, was used to examine the role of PLC in MT-mediated chemotaxis. These experiments showed that pre-incubation with U73122 blocks the chemotactic response in both lymphocytes (Figure 7 A+B) and breast cancer cell models (Figure 7 C+D). These results suggest that PLC signaling is involved in the initiation of MT’s chemotactic response.

MEK signaling cascades are involved in many important cellular processes including differentiation and proliferation\(^{45}\). While previous studies in the Lynes Lab have shown that MT exposure results in short term activation of ERK1/2\(^{25}\), the role of this pathway in MT’s chemotactic response was not known. PD98059, a small molecule inhibitor of MEK, was used to determine the involvement of MEK signaling in MT-mediated chemotaxis. Pre-incubation with PD98059 did not block MT’s chemotactic response in both lymphocytes (Figure 8 A+B) and mammary tumor cell lines (Figure 8 C+D). These results indicate that MEK is likely not involved in MT’s chemotactic signaling pathways.

Finally, the ability of UC1MT to block MT-mediated chemotaxis in mammary tumor cell lines was examined. Previous studies showed that pre-incubation with UC1MT blocks MT-mediated chemotaxis in lymphocytes, and that it has therapeutic potential in the treatment of IBD and diabetes\(^{11,22,25}\). However, the potential of using UC1MT as a therapeutic for breast cancer had not yet been evaluated. Results here show that pre-incubation with UC1MT specifically blocks MT-mediated chemotaxis in mammary tumor cell lines (Figure 9). This suggests that UC1MT may be a valuable therapeutic to inhibit breast cancer metastasis.

Taking the results from all these experiments into account, I have proposed a model for the signaling pathways involved in MT-mediated chemotaxis (Figure 10). I propose that
exogenous MT interacts with CXCR4 (or other unknown receptors) on the surface of the cell. This binding event results in the dissociation of $G_\alpha$ and $G_{\beta\gamma}$. Currently, the role of $G_\alpha$ in MT-mediated chemotaxis is unknown. $G_{\beta\gamma}$ activates signaling cascades involving PLC and the Arp2/3 complex, which results in a chemotactic response. It is of note that $G_{\beta\gamma}$ may initiate additional pathways to result in chemotaxis, however we have not explored these possibilities.

While MEK signaling is known to have important functions in the cell, it is not involved in MT’s chemotactic signaling pathways. Finally, UC1MT can bind to exogenous MT to block MT’s interaction with its receptor and prevent the chemotactic response.

These results have provided valuable information regarding the mechanism by which MT initiates a chemotactic response, which allows us to further understand MT’s role in inflammation and disease processes, and will help us identify other therapeutic targets for UC1MT in the future. Furthermore, the fact that UC1MT blocks MT-mediated chemotaxis in mammary tumor cell lines suggests that UC1MT may inhibit breast cancer metastasis.

Next steps for this project include investigating the role of other chemokine receptors, such as CXCR7, and the role of other common GPCR signaling cascades in MT-mediated chemotaxis. Most importantly, animal studies, likely in a mouse model, must be conducted to determine if UC1MT can inhibit mammary tumorigenesis and metastasis to improve patient outcomes.
Figure 10. Proposed Signaling Pathway of MT-mediated Chemotaxis

Exogenous MT interacts with chemokine receptor CXCR4. The binding of MT to CXCR4 results in the dissociation of $G_\alpha$ and $G_{\beta\gamma}$. The role of $G_\alpha$ in MT-mediated chemotaxis is currently unknown. Results from these experiments suggest that $G_{\beta\gamma}$ initiates downstream signaling cascades, which result in the activation of both the Arp2/3 complex and phospholipase C (PLC). The activation of these complexes initiates cytoskeletal reorganization, which leads to the chemotactic response. It is currently unknown if $G_{\beta\gamma}$ activates other downstream signaling pathways to initiate MT’s chemotactic response. UC1MT can bind to MT to block its interaction with CXCR4 (or other unknown receptors) and inhibit MT’s chemotactic effect. MEK signaling does not appear to play a role in MT-mediated chemotaxis.
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22. Devisscher L, Hindryckx P, Lynes MA, et al. Role of metallothioneins as danger signals


**Figure S1. Dose Response and Cell Viability with AMD3100 Pre-incubation**

MDA-MB-231(A+B) or 419 (C+D) cells were pre-incubated with various concentrations of AMD3100 for 30 minutes at 37°C in 5% CO₂. Media and MT controls were pre-incubated under the same conditions in media alone. After pre-incubation, cell viability (B+D) was calculated using a hemocytometer and trypan blue exclusion staining. The bottom chamber was loaded with either 10µM MT (MT control and AMD3100) or media alone (Media control). Boyden chamber
was assembled using an 8µm (A) or 3µm pore (C) membrane. 8µm pore membranes were coated in 10µg/mL fibronectin at 4°C overnight prior to use. Cells were washed in media, resuspended at 2 million cells/mL, and loaded into the top chamber. Boyden Chamber was incubated for 6 hours (A) or 3 hours (C) at 37°C in 5% CO₂. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001.
Figure S2. Dose Response and Cell Viability with CK-666 Pre-incubation

Jurkat T cells (A+B), Primary Mouse Splenocytes (C+D), MDA-MB-231 cells (E+F), or 419 cells (G+H) were pre-incubated with various concentrations of CK-666 for 1 hour at 37°C in 5% CO$_2$. Media and MT controls were pre-incubated under the same conditions in media alone. The bottom chamber was loaded with either 10µM MT (MT control and CK-666) or media alone (Media control). After pre-incubation, cell viability (B+D+F+H) was calculated using a hemocytometer and trypan blue exclusion staining. Boyden chamber was assembled using an 8µm (E), 5µM (A), or 3µm pore (C+G) membrane. 8µm pore membranes were coated in 10µg/mL fibronectin at 4°C overnight prior to use. Cells were washed in media, resuspended at 2 million cells/mL, and loaded into the top chamber. Boyden Chamber was incubated for 3 hours (A+C+G) or 6 hours (E) at 37°C in 5% CO$_2$. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001, and **** = p ≤ 0.0001.
Figure S3. Dose Response and Cell Viability with U73122 Pre-incubation

Jurkat T cells (A+B), Primary Mouse Splenocytes (C+D), MDA-MB-231 cells (E+F) or 419 cells (G+H) were pre-incubated with various concentrations of U73122 for 30 minutes at 37°C in 5% CO\textsubscript{2}. Media and MT controls were pre-incubated under the same conditions in media alone. After pre-incubation, cell viability (B+D+F+H) was calculated using a hemocytometer and trypan blue exclusion staining. The bottom chamber was loaded with either 10\mu M MT (MT control and U73122) or media alone (Media control). Boyden chamber was assembled using an 8\mu m (E), 5\mu M (A), or 3\mu m pore (C+G) membrane. 8\mu m pore membranes were coated in 10\mu g/mL fibronectin at 4°C overnight prior to use. Cells were washed in media, resuspended at 2 million cells/mL, and loaded into the top chamber. Boyden Chamber was incubated for 3 hours (A+C+G) or 6 hours (E) at 37°C in 5% CO\textsubscript{2}. Cells on the underside of the membrane were enumerated using a light microscope under 10x. One-way ANOVA followed by Tukey’s Multiple Comparisons Test was used to determine statistical significance with * = p \leq 0.05, ** = p \leq 0.01, *** = p \leq 0.001, and **** = p \leq 0.0001.
Figure S4. Cell Viability with PD98059 Pre-incubation

Jurkat T cells (A), Primary Mouse Splenocytes (B), MDA-MB-231 cells (C) or 419 cells (D) were pre-incubated with various concentrations of PD98059 for 1 hour at 37°C in 5% CO₂. Media and MT controls were pre-incubated under the same conditions in media alone. After pre-incubation, cell viability was calculated using a hemocytometer and trypan blue exclusion staining.