RESPONSE OF TRIPLE NEGATIVE BREAST CANCER CELLS TO EXTRACELLULAR NUCLEOTIDES

by

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ABSTRACT

Inflammatory breast cancer (IBC) and triple negative non-IBC are two highly aggressive forms of breast cancer that are difficult to treat and have high incidences of recurrence. Most IBC are also triple negative in nature and have a molecular profile that classifies them as basal cell type. Despite some similarities, these distinct forms of breast cancer have phenotypic differences that set them apart. The P2Y receptors are a class of G protein coupled receptors that control key cellular processes such as growth, proliferation, and migration in both normal and pathological states. The eight known subtypes of the P2Y receptors have been identified in several cancers and can vary in expression depending on the cancer type. **In this study, we hypothesize that P2Y receptors promote the aggressive phenotype of both triple negative inflammatory and non-inflammatory breast cancer.**

As the exact receptor subtypes have been characterized in only a few cancer cell lines, in this study we compare RNA expression profiles of P2Y receptors from a triple-negative inflammatory breast cancer cell line (SUM149) and triple-negative non-inflammatory breast cancer cell lines (GI101A and GILM2, an isogenic progression of the GI-101A cells).

Once the receptor subtypes are found, the specific agonists for each could be identified through the use of an extensive database that details the

pharmacology of these receptors. We demonstrated that treating the cells with the relevant agonists had a differential effect with respect to proliferation and invasion. Furthermore, in the process of identifying the most effective ligands, we discovered that adenosine had a considerable negative effect on growth in the SUM149 cells that was not seen in the non-IBC cell lines. This data led us to believe that there may be a difference in adenosine receptor expression on these cells. Like the purinergic receptors, adenosine receptors have been implicated in the development and progression of various types of cancers. We show that our three cell lines in fact express only the same adenosine receptor subtype, A_1. It was noted, however, that the levels of RNA expression vary between them. This may, in part, explain some of the differential responses we observed among these cells.

The treatment of cancer with exogenous nucleotides like ATP has shown some initial promise in the clinic. Considerably aggressive, inflammatory breast cancer and triple negative breast cancers are particularly deadly because of the high rate of treatment failure and recurrence. This study examines the response of triple negative breast cancer cells to extracellular nucleotides/nucleosides. Our data show the antiproliferative effects of ATP and its derivatives, giving hope that, perhaps one day, it may be used as an adjuvant therapy for these devastating cancers.

Chapter 1

INTRODUCTION

1.1 Breast Anatomy and Physiology

The human breast is a complex glandular structure comprised of skin, connective tissue, and breast tissue. The breast tissue includes glandular epithelial cells, ductal epithelial cells, and lymphatic vessels. Each breast lies between the second and sixth ribs and between the sternum and the midaxillary line (Harris et. al, 2009). The functional unit of the breast is the lobe, comprised of approximately 20 to 40 lobules. Each lobule consists of 10 to 100 alveoli – the site of milk production and storage (Figure 1.1) Milk ejection is stimulated by suckling of the newborn at the nipple, resulting in contraction of the myoepithelial cells surrounding the alveoli and milk ducts (Neville, 1998). Milk is carried out of the alveoli and towards the nipple by ductal segments. These segments converge into ten major collecting ducts, which open at the nipple (Harris et. al, 2009). One of the last stages of normal breast maturation is involution, or the gradual shrinking of the mammary glands. This process usually occurs around the age of 35 (Harris et. al, 2009).

The development of the adult female breast is stimulated by estrogens and progesterone, although other hormones are thought to play a minor role. The

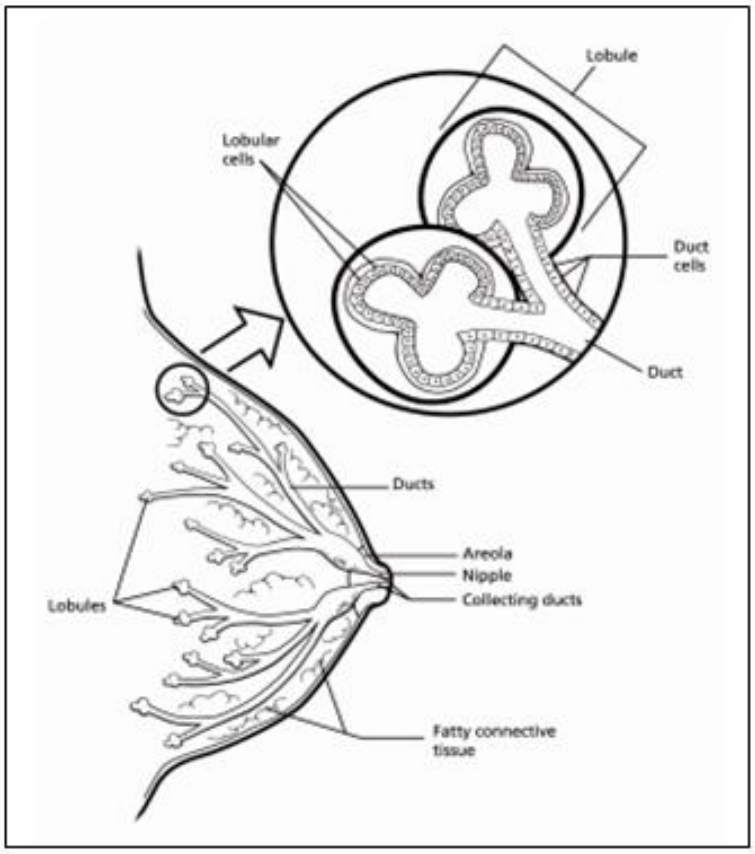


Fig 1.1 – Microanatomy of the normal breast. Note the lobular and ductal cells and their arrangement within the breast. The collecting ducts converge at the nipple where milk letdown occurs. Reprinted by the permission of the American Cancer Society, Inc. from www.cancer.org.

differentiation of the breast parenchyma from adolescent tissue into adult is thought to be complete by 14 to 15 years. Exposure of the breast tissues to estrogen results in the proliferation of the mammary glandular epithelium. The tissue becomes multilayered and starts to form buds and papillae. The cells of this multilayered tissue are comprised of three subtypes: superficial (luminal) cells, basal B (chief) cells, and the previously mentioned myoepithelial cells (Harris et. al, 2009; Vorherr, 1974).

1.2 Breast Cancer

Statistically, one in eight U.S. women will be diagnosed with some form of breast cancer in their lifetime (breastcancer.org, 2011). It is the second most prevalent cancer in women, with nearly 290,000 women having been diagnosed in 2011. Of these patients, approximately 40,000 will succumb to the disease (breastcancer.org, 2011). Breast cancer can be defined broadly as the uncontrolled growth of cells that comprise the breast tissue (see Figure 1.2). Typically, these cancerous cells arise from the ducts (ductal carcinoma), which carry milk from the breast lobule to the nipple; or less commonly, the neoplastic cells may originate in the lobes (lobular carcinoma), the glands responsible for milk production. Even more infrequent is breast cancer arising from the myoepithelial cells, however they have been found in different benign breast tumors and certain breast sarcomas (Lahkani, 2001). Some breast cancers remain confined to the ducts (ductal carcinoma in situ) or the lobes (lobular carcinoma in situ)

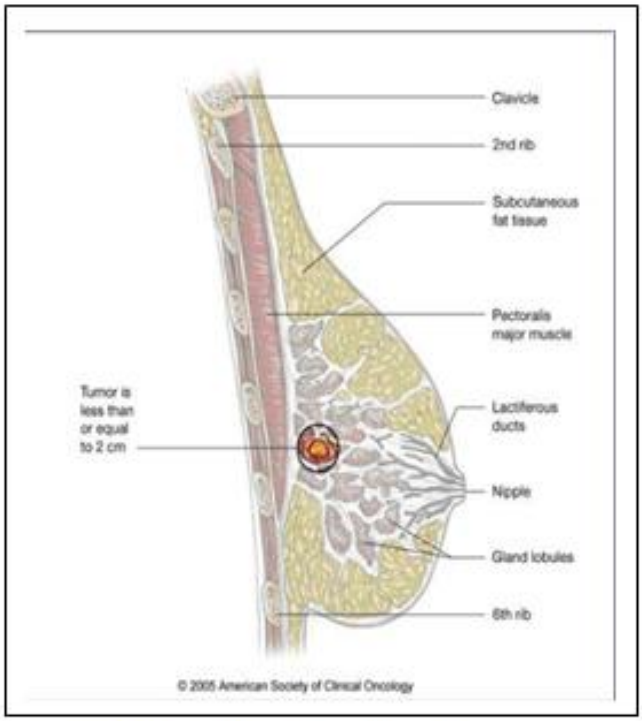


Figure 1.2: Classic presentation of breast cancer. More common forms of breast cancer typically present with a small lump within the breast. Adapted from http://www.vchangeu.com/stages.html

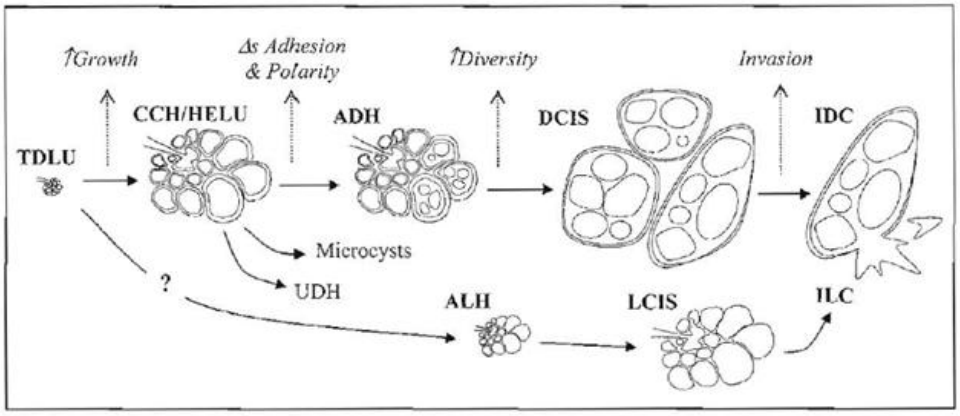


Figure 1.3: Model of breast cancer progression. Normal terminal duct lobular unit (TDLU) progresses to columnar cell hyperplasia (CCH) or hyperplastic enlarged lobular units (HELU). Eventually this leads into atypical ductal hyperplasia (ADH), ductal carcinoma in situ, and eventually the disease becomes invasive (IDC). A similar progression can occur within lobular cells. Adapted from Diseases of the Breast, 4th ed. 2010.

where they originated. Ductal carcinoma in situ (DCIS) is the more common of the two, comprising approximately 83% of all in situ cases (ACS Breast Cancer Facts and Figures, 2011-2012). The prognosis tends to remain favorable for this early stage of breast cancer – most women tend to be cured (ACS, 2011). However, most breast cancers tend to be invasive (infiltrating), accounting for 80% of all cases (See Figure 1.3)(ACS, 2011). Invasive ductal carcinoma (IDC) occurs when the neoplastic cells have broken through the ductal or lobular membrane to infiltrate the surrounding tissue. The potential for these infiltrating cells to metastasize to other parts of the body via the bloodstream or lymphatics is high, thus a diagnosis of IDC has its concerns.

The severity of IDC is influenced by the stage of disease. In the United States and some European countries, the staging of breast cancer is the TNM system and relies on three critera: tumor size, lymph node involvement, and the presence of metastasis. Staging ranges from stage 0 to stage IV - typically the higher the stage number, the more aggressive the cancer (see Figure 1.4).

In addition to the more commonly diagnosed breast cancers, there exist several other forms. Most notable is inflammatory breast cancer (IBC). IBC accounts for 1-3% of all breast cancer cases according to recent SEER data, however earlier estimates suggest that the rates may be closer to 5-7% (ACS, 2011). The differences in the numbers come from the SEER redefinition of IBC. While neither estimate may be correct, the numbers in both cases are thought to be notoriously low due to lack of accurate reporting. The name inflammatory breast cancer comes from the inflamed

Stage	T (tumor size)	N (node status)	M (metastasis)
0	No evidence of tumor		
I	T-1	N-0	M-0
II	T-1 T-2 T-3	N-1 N-0 or N-1 N-0	M-0
III	T - any size T-3 T-4	N-1 N-0 or N-1 N-0	M-0
IV	T - any size	N - any involvement	M-1

Fig 1.4: TNM staging system for the diagnosis of breast cancer.
Adapted from www.breastcancer.org

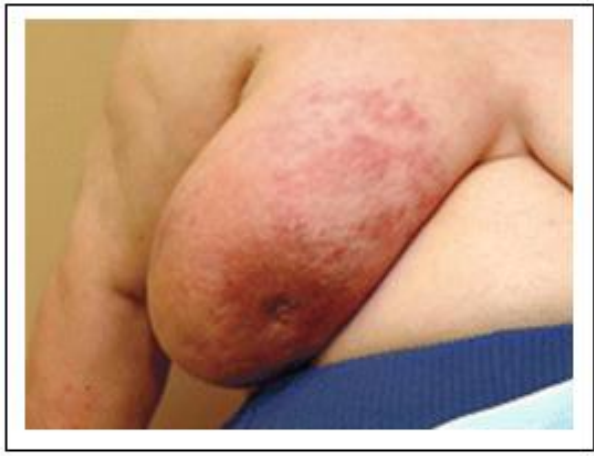


Fig 1.5: Inflammatory breast cancer. Note the primary skin changes, including redness, swelling, and nipple retraction. (adapted from http://www.cfp.ca/content/55/1/25.full, 2012)

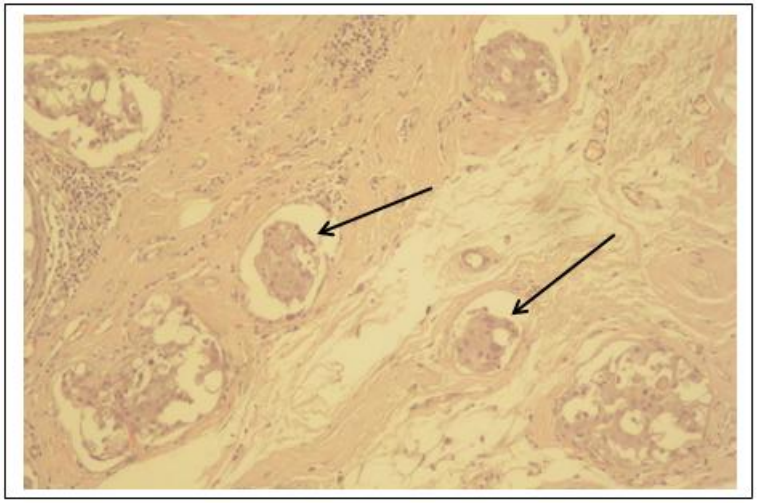


Fig 1.6: Tumor emboli within the dermal lymphatics of the breast. Tumor emboli form and block the dermal lymphatics, causing the primary skin changes that are the hallmark of inflammatory breast cancer.

appearance of the breast that can resemble mastitis; however, it is not an inflammatory process. The main symptoms of IBC are primary skin changes, e.g. swelling, itching, reddening of the skin, warmth, tenderness, pitting of the skin resembling an orange peel (peau d'orange), as seen in Figure 1.5. It is thought that these skin changes are due to the blockage of the dermal lymphatics by tumor emboli (Figure 1.6). Other symptoms include nipple retraction and swollen axillary lymph nodes. These can develop quickly over weeks or months. Because of its unique presentation, the lack of a palpable mass and the rapid onset, often it is difficult to recognize IBC. As a result, physicians can waste precious time with antibiotic treatment. Obviously ineffective, misdiagnosis can be a matter of life or death due to the aggressive, highly metastatic nature of IBC. Most patients do not initially present with any detectable lumps in the breast, thus by the time a correct diagnosis is made, the cancer has already progressed to stage III or IV. Treating IBC is different than non-IBC in that surgery is not the first treatment. This approach, known as neoadjuvant therapy, recommends that systemic chemotherapy be given in an attempt to shrink the tumor, reduce swelling and improve the "inflammation" of the breast (breastcancer.org). Chemotherapy often includes anthracyclines combined with a taxane. Additional therapy may include trastuzumab or lapatinib if the cancerous tissue stained positive for HER2/neu, an epidermal growth factor receptor (EGFR) family member. This monoclonal antibody works by binding to the HER2 receptor, blocking signaling cascades that induce cell proliferation. If clinical response is observed with skin punch biopsies showing a lack

of tumor emboli, patients then undergo a modified radical mastectomy, a procedure that involves the total removal of the affected breast and all lymph nodes under the arm. The aggressive nature of this disease requires an aggressive treatment – lumpectomy or a partial mastectomy is not recommended because IBC typically involves the entire breast. Sentinel lymph node dissection, where only a few lymph nodes are removed, is not reliable and therefore is not routinely performed (ACS, 2011). After surgery, radiotherapy is often the next stage of treatment. Radiation is a standard treatment for IBC as it reduces the odds of recurrence (ACS, 2011). Targeted areas typically include the breast, the chest and chest wall, as well as the under arm and collarbone to include lymph nodes not removed by surgery (breastcancer.org). Once radiation is complete, doctors typically continue treatment, which may vary based on the patient's response thus far. Inflammatory breast cancer requires a significant amount of treatment due to its aggressive nature and high incidences of recurrence. Further treatment may include additional chemotherapy, hormonal therapy (if the tissue is positive for estrogen receptor [ER] or progesterone receptor [PR]) and trastuzmab/lapatinib (if the tissue is HER2/neu positive). However, the majority of inflammatory breast cancer cases are triple negative (IBC Research Foundation). Triple negative breast cancer (TNBC) refers to the lack of receptors for estrogen and progesterone within the cell, as well as HER2/neu (EGFR2/ERB2) receptors that are on the cell surface. Conventional non-IBC can be triple negative, comprising about 10-20% of all breast cancers. TNBCs are known to be highly aggressive, metastatic

and have a high incidence of recurrence. The standard of care usually consists of surgery and radiation. Due to the lack of hormone and growth factor receptors, adjuvant therapy, or therapy used after the primary therapy to prevent recurrence, such as hormone therapy and drugs that target HER2, are ineffective. Chemotherapy, however, may still be effective (ACS, 2011; Mayo Clinic, 2010).

1.3 Classification of Breast Cancer

As science and medicine have progressed, the means of classifying and diagnosing cancer has become an intricate process. Determining the molecular signatures of a cancer is the new approach to treatment as "personalized medicine" – or treatment based on the individual characteristics of the cancer – has eclipsed the old approach of treating cancers as though they behave similarly. Today, six molecular subtypes of breast cancer have been identified: the luminal A and luminal B subtypes (both estrogen receptor [ER] alpha positive), the normal-like HER-2/neu positive subtypes, and the basal subtypes (the triple negative breast cancers) (Yalcin-Ozuysal, 2009). The luminal (superficial) A cells contain numerous ribosomes and contain high levels of RNA in their cytoplasm, indicating increased protein synthesis. The proliferation of these cells is driven primarily by estrogen, hence the expression of ER within the cell. Luminal A cells also may express progesterone receptors (PR), but rarely express the EGFR2 (HER2/neu). These cells are typically found in low to

moderate grade breast cancer tumors. Luminal B cells appear like luminal A cells in that they tend to be ER positive and/or PR positive. These cells are dividing actively and may be why breast cancers with this molecular signature tend to have a poorer prognosis (Vorherr, 1974).

The basal cells, or "chief cells," are considered to be the main structural element of the mammary epithelium as they typically comprise the deeper layers of the mammary tissue. Basal cells are held together by desmosomes, which mediate cell-cell adhesions and provide stability to the tissue (Lodish et al, 2007). These cells tend to have clear cytoplasm with a rounded nucleus and appear like cells that line the mammary ducts (Vorherr, 1974; Harris et al, 2009). The majority of the basal cell type are ER/PR and HER2 negative, known as triple negative breast cancer (TNBCs) (Harris et al, 2009). Basal-like tumors tend to be the most aggressive type of breast cancer in that they have shorter relapse-free survival times and shorter survival times compared to other subtypes (Schottenfield et. al, 2006). TNBCs are basal-like tumors and represent the most difficult types of tumors to treat. With a lack of targets for adjuvant therapy, novel targets need to be identified.

1.4 Nucleotide/Nucleoside Receptors

G protein-coupled receptors (GPCRs) comprise one of the largest classes of transmembrane receptors. More than 500 mammalian genes are known to encode

receptors for steroid and peptide hormones, paracrine factors and neurotransmitters, among others. The diversity of GPCR signal transduction is compounded by the fact that different signaling cascades can be initiated from the same receptor and the same ligand based on the Gα protein subtype bound to the receptor (Alberts et al, 2008). A myriad of cellular responses can result from these signaling cascades, including growth, differentiation, proliferation, and apoptosis. These processes, while essential for a normal cell, become altered in different pathophysiological states, including cancer.

1.4.1 Purinergic Receptors

Purinergic receptors are a family of cell surface receptors involved in a host of cellular processes, including proliferation, differentiation, migration, and apoptosis. The notion that nucleotides can function as extracellular signaling molecules is a fairly recent proposal, which shattered the widely-held belief that they existed only as intracellular energy molecules (Burnstock, 1997). However, extracellular adenine molecules and their effect on cardiac tissue were first described in 1929 by Drury and Szent-Gyorgyi. Since then, several studies were carried out to examine actions of purine nucleotides. It was in 1972 that Burnstock suggested that ATP was "a transmitter involved in non-adrenergic, non-cholinergic (NANC) nerve-mediated responses of the smooth muscle in the GI tract and bladder" (Burnstock, 1997). The

defining of two major subgroups of purinergic receptors did not occur until 1978 when Burnstock divided the P1 purinoreceptors – selective for adenosine – and the P2 purinoreceptors – selective for nucleotides. A year later, P1 receptors were further subdivided into A1 and A2 by Van Calker et al. and Londos et al. In 1985, the subdivision of the P2 receptors was proposed by Burnstock and Kennedy – P2 now included P2X and P2Ysubtypes. In the years following the development of the nomenclature, it has been shown that the P2Y receptors were G-protein-coupled receptors while the P2X subtypes were ligand-gated ion channels (Burnstock, 1997).

To date, there are seven recognized P2X receptor subtypes – $P2X_1$ – $P2X_7$. Each receptor consists of three P2X subunits, each of which contains two transmembrane domains connected by a richly N-glycosylated extracellular loop that always contains ten cysteines. The structure of these receptors is said to resemble the inward rectifier K^+ channel and the epithelial Na^+ channel (Figure 1.7). P2X subunits are capable of forming homodimers and heterodimers. Upon activation, P2X receptors mediate Na^+ and Ca^{2+} influx and K^+ efflux, resulting in membrane depolarization and subsequent voltage-sensitive channel activation (Burnstock1997; White, 2006).

P2Y receptors differ in that they are coupled to heterotrimeric G-proteins along the inner leaflet of the cell membrane. Eight P2Y receptor subtypes have been identified thus far – $P2Y_1$, Y_2, Y_4, Y_6, and Y_{11} – Y_{14}. Each subtype has an extracellular N-terminal domain, seven transmembrane domains, and an intracellular C-terminal domain (Figure 1.7)(Burnstock, 1997). P2Y receptor activation can vary depending on

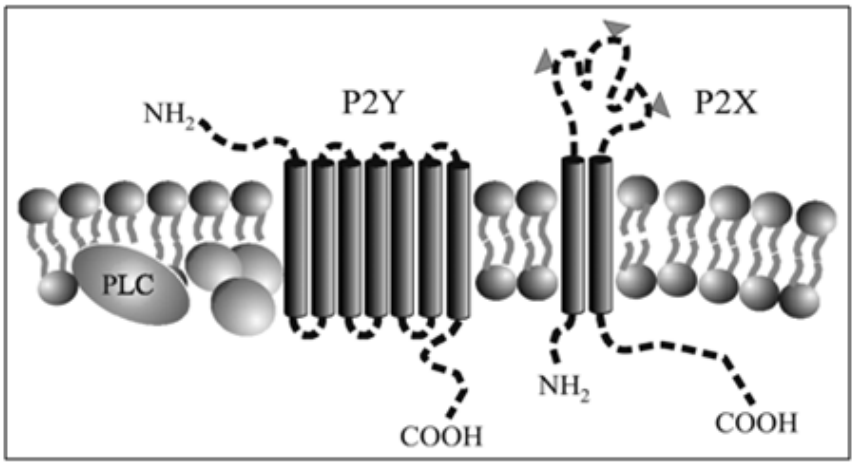


Fig 1.7: P2Y and P2X receptors. P2Y receptors are 7-pass transmembrane G protein coupled receptors. P2X receptors are ligand gated ion channels (adapted from http://physiologyonline.physiology.org/content/18/1/12.full, 2012)

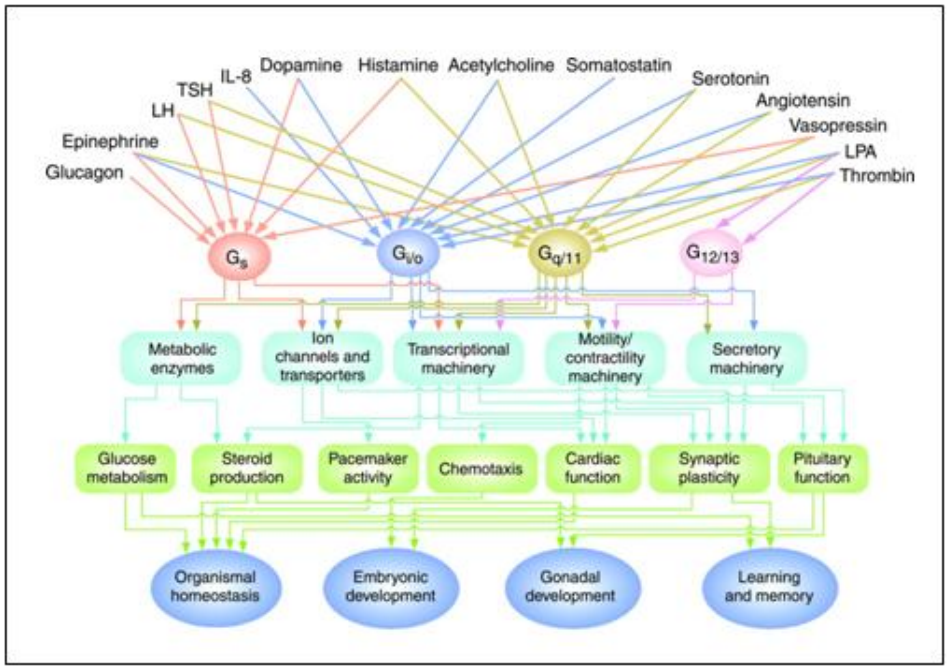


Fig 1.8: The various signaling pathways of the Gα proteins. From Neves S; Ram P.T.; Iyengar R. (2002) *G protein pathways.* 296 (5573). Reprinted with permission from AAAS.

the receptor subtype and also may be depend on the tissue in which these receptors are expressed. Additionally, different Gα subunits are capable of coupling to the P2Y receptor subtypes, including $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_s$ and $G\alpha_{12/13}$ (Abbracchio et al., 2011 – IUPHAR database). Signaling from each subunit can yield different cellular responses, e.g. proliferation, differentiation, migration and invasion, depending on the particular cellular scaffolding and the signaling proteins involved (Figure 1.8).

To date, $G\alpha_{q/11}$ has been shown to couple with several P2Y receptor subtypes, including $P2Y_1$, Y_2, Y_4, Y_6, and Y_{11} (Abbracchio et al., 2011). Classically, $G\alpha_{q/11}$ is known to activate phospholipase C (PLC), leading to the formation of the second messengers inositol 1,4,5-triphospate (IP_3), a molecule responsible for releasing Ca^{2+} from intracellular stores, and diacyl glycerol (DAG), a lipophilic molecule that activates protein kinase C (PKC). The downstream signaling of PKC can influence cellular growth, metabolism, and proliferation (Alberts et al, 2008; Lodish, 2003).

$G\alpha_{i/o}$ is another G-protein that is associated with a number of P2Y subtypes, including $P2Y_2$, Y_{12}, Y_{13}, and Y_{14} (Abbracchio et al., 2011). $G\alpha_{i/o}$, an inhibitory G protein, binds directly to adenylyl cyclase (AC), inhibiting the production of cyclic adenosine monophosphate (cAMP). Additionally, $G\alpha_{i/o}$ can regulate ion channels (Alberts et al, 2008). $G\alpha_s$ is similar in that it has an effect on AC and cAMP levels, however it is a stimulatory G-protein, and unlike $G\alpha_{i/o}$, causes an increase in cAMP. This second messenger has been shown to activate cyclic-AMP-dependent protein kinase (PKA), a signaling molecule that is capable of various cell functions, including

activation of gene transcription (Alberts et al., 2008; Lodish, 2003). $G\alpha_s$ has been linked to $P2Y_{11}$ (Abbracchio, 2011).

A fourth Gα subtype that has been shown to couple with P2Y receptors is $G\alpha_{12/13}$ – specifically $P2Y_2$ and Y_6 subtypes (Burnstock et al, 2012). Like the other subtypes previously mentioned, $G\alpha_{12/13}$ has various effectors, many of which are tissue and cell-specific. However, $G\alpha_{12/13}$ is interesting in that it primarily activates Rho guanine exchange factors, or RhoGEFs. These molecules are potent activators of RhoA , which, in conjunction with its effectors, induce signaling that leads to actin cytoskeletal rearrangement, cell migration, and invasion. Activated $G\alpha_{12/13}$ has also been shown to bind to certain cadherins, a protein involved in cell-cell and cell-ECM interactions (Williams, 2011).

1.4.2 Adenosine Receptors

Adenosine receptors are a group of G-protein coupled receptors that are expressed widely in a number of human tissues. As their name suggests, the dominant agonist to these receptors is adenosine although the IUPHAR database reports other important compounds as well. The release of adenosine from cells occurs through the process of metabolism and is generated by the breakdown of ATP. Currently four subtypes of adenosine receptors have been identified: A1, A_{2A}, A_{2B}, and A3. These subtypes may be coexpressed in the same tissue (Merighi et al, 2003). The G proteins

to which adenosine receptors couple vary depending on the receptor subtype and some subtypes may couple to more than one G protein. The A_1 and A_3 receptors have been shown to couple to $G\alpha_{i/o}$, inhibiting adenylyl cyclase (AC). The inhibition of adenylyl cyclase blocks the production of cAMP thereby preventing the activation of PKA. PKA, when activated, is responsible for various cellular processes including the initiation of gene transcription (Alberts et al, 2008).

The A_{2A} and A_{2B} receptors couple primarily to $G\alpha_s$, stimulating AC and ultimately activating cAMP and phospholipase A, and secondarily to $G\alpha_{q/11}$, triggering a rise in intracellular Ca^{2+} and also stimulating phospholipase C (Fredholm et al., 2012).

1.5 Nucleotide/Nucleoside Receptors and Cancer

It has been nearly forty years since the concept of nucleotides functioning as extracellular signaling molecules was first proposed by Burnstock. Initially, this supposition was met with much resistance, as it was believed that ATP and its derivatives served only as energy molecules and was "too ubiquitous a molecule to be involved in selective extracellular signaling" (Burnstock, 2006). Since then the field of purinergic signaling has been studied extensively – its role in normal cellular function and how it contributes to the development of disease.

1.5.1 P2 Receptors and Cancer

Numerous cellular functions are said to be derived from the signaling pathways initiated by P2 receptors. While there are two distinctive families of P2 receptors – P2X and P2Y – they share similarities in that the downstream responses of activation cause significant physiological changes to the cell. The P2X receptors are ligand-gated ionotropic channels that, upon activation by ATP, cause a rapid influx of Na^+ and Ca^{2+} and an efflux of K^+. Membrane depolarization and the rise of Ca^{2+} activates signaling cascades that initiate various physiological processes like nerve impulses, pain sensation, and movement (Young, 2009). Many tissues throughout the human body express P2X receptors, especially in cells of epithelial origin, e.g. skin, gut, kidney. Expression of these receptors also has been identified in cancerous tissues and/or cell lines, including skin, colorectal, oropharyngeal, cervical and breast (White and Burnstock, 2006).

Similarly, the expression of the G-protein coupled P2Y receptors in normal and cancerous tissues has been noted. Unlike the P2X receptors, P2Y receptors couple to small heterotrimeric G proteins – the Gα protein contributing to the majority of variation in signaling cascades. These pathways lead to changes in cellular functions, including proliferation, differentiation, and migration (White and Burnstock, 2006). Although P2X receptors have a much lower affinity than the P2Y receptors, both are capable of stimulation by ATP, and in the case of the P2Y receptors, other nucleotides

and their derivatives. For instance, P2Y receptor subtypes $P2Y_2$, Y_4, Y_6, Y_{11}, and Y_{14}, can be activated by UTP in addition to ATP. Furthermore, the presence of ectonucleotidases – enzymes on the cell surface that break down the triphospate forms of these molecules into their mono- and diphosphate forms – add to the complexity of purinergic signaling (White and Burnstock, 2006). While ATP initially may be present in the extracellular environment, it is uncertain whether it may be acting on a particular receptor in its native form or if it is cleaved by these enzymes prior to binding to its receptor. Characterization of receptor and ectonucleotidase expression in human tissues is currently underway.

The use of ATP as a therapeutic agent for the treatment of cancer in animal models first occurred in 1983. Daily intraperitoneal injections of ATP into mice reduced tumor growth and inhibited weight loss (cachexia), thereby increasing survival (Rapaport et al, 1983). In humans, ATP has demonstrated an antiproliferative effect in numerous types of cancers, including breast. Additionally, studies show that administration of ATP in humans reduces tumor resistance to chemotherapy treatment. The use of ATP as an antineoplastic agent has been demonstrated in both phase I and phase II clinical trials, thus establishing it as a promising treatment option and opening the door to the use of similar nucleotides as a method of treatment (White and Burnstock, 2006).

1.5.2 Adenosine Receptors in Cancer

It has been well-established that adenosine plays a significant part in cell signaling throughout the body. The receptor subtypes expressed in a particular tissue control the response to adenosine based on the particular signaling cascade activated by ligand binding. For instance, the relaxation of the vasculature smooth muscle is mediated by adenosine and it binding to the A_{2A} receptor subtype and subsequent activation of $G\alpha_s$ and stimulation of cAMP. Conversely, the A_1 receptor expressed in cardiac tissue is coupled to $G\alpha_i$, leading to K^+ channel opening and membrane hyperpolarization (Klabunde, 2007).

The role of adenosine in the development and progression of cancer has been reviewed extensively due to its involvement in angiogenesis, cellular proliferation and immune evasion. In the normal cellular environment, extracellular levels of adenosine remain in the low nanomolar range. However, in times of cellular stress like hypoxia, these levels can rise to micromolar levels due to cellular production and breakdown of ATP. Similar hypoxic environments exist in tumors due to rapid, aberrant cell growth and the inability of these cells to maintain adequate blood supply. It has been reported that adenosine levels in the extracellular fluid around tumors is significantly higher than that of normal tissue (Merighi et al, 2003). Of particular concern is that these increased levels of adenosine in the tumor environment are known to have a negative effect on the ability of the immune system to recognize and fight the cancerous cells.

In colon cancer it was demonstrated that the endogenous levels of adenosine interfered with the activation of killer T-cells as well as the adhesion of murine spleen-derived activated killer lymphocytes to colon adenocarcinoma cells (Gessi et al., 2010).

On the other hand, various in vitro studies have demonstrated that adenosine is responsible for mediating apoptosis in a myriad of different cell types including rat brain astroglial cells, arterial smooth muscle cells and several human cancer cell lines. Other studies have recorded that treatment with adenosine reduces cell proliferation. Perhaps these varying effects are due to the number and type of adenosine receptor subtypes expressed in a particular tissue. Or it could be based on a differential response based on the particular tissue in question. Regardless, it is clear that the differential effect of adenosine is an avenue of cancer research that needs additional exploration.

Chapter 2

MATERIALS AND METHODS

2.1 Cell Culture

The experiments were performed on three breast cancer cell lines. The triple negative non-IBC cell lines, GI101A and GILM2, were obtained from Dr. Janet Price at MD Anderson Cancer Center, Houston, TX. GI101A cells were isolated from a recurrent primary breast cancer in a patient that subsequently developed lung metastasis. GILM2 were developed as an isogenic progression of GI101A injected into mice. The lung metastases were removed and plated in culture dishes. The resulting cells were named GILM1. GILM2 were produced from the second in vivo cycle. Both cell lines were grown in DMEM (Meditech, Inc. Manassas, VA) with 10% FBS (Atlanta Biologicals, Lawrenceville, GA) and 1% Pen-Strep (Mediatech, Inc.). The inflammatory breast cancer line SUM149 was originally derived from a primary IBC and grown in Ham's F-12 (Mediatech, Inc.) with 5% FBS (Atlanta Biologicals), 1% Pen-Strep (Mediatech, Inc), 1% antibiotic/antimycotic (Mediatech, Inc.), 1% Insulin/Transferrin/Selenium cocktail (Gibco, Carlsbad, CA), 1% L-glutamine (Mediatech, Inc.), and 1µg/mL hydrocortisone (Sigma-Aldrich, St. Louis, MO).

Unless specifically stated, all cells were maintained at 37^{o}C in a humidified atmosphere of 95% air and 5% CO_2.

2.2 Reverse Transcriptase (RT)-PCR

Reverse transcriptase PCR was conducted to identify which, if any, P2Y and adenosine receptor subtypes were expressed in each cell line. Total RNA was extracted from each cell line using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Optimal phase separation was achieved using 2mL Phase Lock Gel (heavy) tubes (Eppendorf, Hamburg, Germany). Total RNA was DNase-treated using Ambion DNA^{TM} Kit (Ambion, Austin, TX) according to the manufacturer's guidelines. RNA quality and concentration was analyzed spectrophotometrically. RNA (1µg) was reverse transcribed in a final volume of 12uL using the Maloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA) and then stored at -20^{o}C. Primers of each receptor subtype and GAPDH as positive control can be found in Table 3.1. PCR was carried out using the GoTaq® Hot Start PCR kit (Promega, Madison, WI). 5µL of the reaction mixture was electrophoresed on 1.5% agarose gel and visualized with ethidium bromide using a 100bp ladder (Fermentas, Burlington, Canada) to estimate band sizes.

2.3 Dose Response Assays

Assays were performed on SUM149 cells in tissue culture-treated 24-well plates (Greiner, Austria). 5000 cells were plated and given 24h to attach. ATP, ADP, adenosine, 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate (Sigma, St. Louis, MO), UTP (Calbiochem, Darmstadt, Germany), ATP combined with apyrase (Sigma, St. Louis, MO) and suramin (FBA pharmaceuticals, West Haven, CT) were chosen as agonists based on the mRNA expression profile of P2Y and adenosine receptors for the cell line. Nucleotides were applied in half and full log dilutions ranging from 1μM to 3mM every day for seven days, performing half-media changes with each dosing. After treatment, MTT (methylthazoltetrazolium)(Invitrogen, Carlsbad, CA) assays were conducted to assess cell proliferation relative to non-treated cells. 5mg/mL of MTT reagent was added to each well and incubated for 3h. Stop solution (20% SDS in 50% dimethyl formamide in dH2O) was added to each well. Plates were placed on orbital shaker for 15 min and absorbance was read at 560nm on a PolarStar Optima plate reader from BMG LabTech.

2.4 Focus Formation Assay

GI101A and GILM2 cells were plated separately on tissue culture-treated 6-well or 12-well dishes and allowed to adhere for 24h. Agonists (ATP, ADP,

adenosine, 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate, ATP in combination with apyrase, and UTP) were determined based on the mRNA expression profile of P2Y and adenosine receptors for the cell lines. As 250µM was the approximate IC_{50} value for ATP and ADP when applied to SUM149 cells, this concentration of nucleotide was applied to the GI101A and GILM2 cells every day for seven days, performing half-media changes upon treatment. After treatment, cells were removed from the plate using trypsin (Mediatech, Manassas, VA) and total cell number for each well was obtained. Limiting dilutions of each cell suspension were made (1:500 and 1:1000) and plated in a 12-well plate in triplicate and incubated for ten days. Subsequently, media was aspirated and cells were washed in PBS and stained with 0.4% crystal violet in 20% methanol for 1h at RT. Plates were rinsed in dH_2O and cell colonies quantified under a microscope. Cell groups >10 cells were considered colonies.

2.5 Invasion Assay

The invasive capabilities of GI101A, GILM2 and SUM149 cells were assessed using a Matrigel™ Invasion Chamber 24-well plates with 8µm pore PET (polyethylene terephathalate) inserts (BD, Bedford, MA) according to the manufacturer's guidelines. Cells were removed from the flasks using trypsin (Mediatech, Manassas, VA) and centrifuged. The resulting pellet was washed using

1X PBS to remove traces of trypsin and serum proteins. 25,000 cells were counted and plated in triplicate inserts with serum-free medium. 750µL DMEM medium containing 10% FBS and 1% Pen-Strep was placed in each well as a chemoattractant. After seeding, cells were treated in triplicate with 250µM of agonist or left untreated in normal medium. Plates were placed in the incubator for 24h. Non-invading cells were removed using a cotton swab and remaining cells were stained using 0.4% crystal violet in 20% methanol for 60 min. Inserts were rinsed thoroughly in dH_2O to remove dye and were allowed to dry for 24h. The number of invading cells was assessed using Volocity software.

Chapter 3

P2Y RECEPTOR EXPRESSION IN TRIPLE NEGATIVE BREAST CANCERS AND THE EFFECT OF P2Y AGONISTS ON CELL PROLIFERATION AND INVASION

3.1 Introduction

The phenotypic differences between triple negative inflammatory breast cancer (IBC) and the non-inflammatory breast cancers have been reported extensively (Kleer, et al, 2002; Radunsky and van Golen, 2005; Joglekar M. and van Golen, K.L., 2012). The molecular signatures of metastatic cells of these two types of breast cancer differ in certain cell membrane proteins. For instance, caveolin-1 and E-cadherin are gained with progression of inflammatory breast cancer but lost in the non-IBC cancers. It is believed that these markers are required for tumor emboli formation in the dermal lymphatics. Additionally, the majority of IBC cases have shown that metastatic cells preferentially spread to the visceral organs, e.g. liver, lung while triple negative non-IBC typically form distant metastases in the bone (Radunsky and van Golen, 2005).

It is the phenotypic differences between these two distinct forms of breast cancer that led us to believe that there existed a differential expression of P2Y receptor subtypes between our triple negative IBC (TN-IBC) cell line SUM149 and the triple negative non-IBC (TN non-IBC) cell lines GI101A and GILM2. P2Y

receptor agonists are predominantly nucleotides like ATP, UTP, and derivatives thereof. The signaling pathways activated by receptor stimulation by these nucleotides control cellular processes like proliferation, differentiation, migration and apoptosis. Therefore, it is believed that P2Y receptors may play a role in the development and progression of cancer (White and Burnstock, 2006; Rumjahn, 2006; Buxton, 2010).

Using reverse transcriptase-PCR (RT-PCR), I identified the P2Y receptor subtypes expressed in breast cancer cells. Based on the P2Y receptor subtypes shown to be expressed in our three cell lines, I identified potential receptor agonists of the P2Y receptor subtypes using the IUPHAR database (http://www.iuphar-db.org/DATABASE/FamilyMenuForward?familyId=52) (Burnstock, 2012). The database provided an extensive list of potential agonists for each receptor subtype; however, I chose to limit our selection to molecules that exist or are formed endogenously, e.g. ATP. The notion that nucleotides like ATP can serve as signaling molecules as well as energy molecules was not widely accepted when first suggested by Burnstock 40 years ago. Since that time, there has been extensive research into cellular receptors that bind nucleotides, particularly P2X and P2Y receptors (Abbracchio, 2006; Burnstock, 2000, 2006; White, 2006). Further studies have illustrated the physiological effects of nucleotides on both normal and cancerous cells. Some of these effects include changes in proliferation (Bilbao, 2010), invasion (Buxton, 2010), and angiogenesis (Rumjahn, 2007). In order to prove my overall hypothesis that P2Y receptors contribute to the aggressive phenotype of triple negative IBC and non-IBC cancer,

I sought to study the effect of nucleotide treatment on the proliferation of SUM149, GI101A and GILM2 cells.

3.2 Results

3.2.1 P2Y Receptor Expression in Triple Negative Breast Cancer Cells

The cDNA for each cell line was used in the RT-PCR reaction with primers for each of the eight subtypes of P2Y receptors. $P2Y_6$ and $P2Y_{14}$ had two different sets of forward primers to account for different splice variants (see Table 3.1) The RT-PCR products were then separated in 1.5% agarose gel for 45 minutes at 100 volts. RT-PCR images (Figure 3.1, A-C) show the receptor subtypes expressed for each cell line. As hypothesized, there exists a differential expression of P2Y receptors for each cell line. SUM149 cells show expression of $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$. GI101A cells express $P2Y_2$, $P2Y_6$, and $P2Y_{11}$ while the isogenic progression line of GI101A, the GILM2 cells, express only $P2Y_2$ and $P2Y_6$.

3.2.2. Dose Response to Nucleotide Treatment with ATP, ADP, and UTP

Based on the P2Y receptor subtypes identified by RT-PCR for each of the cell lines, the nucleotides ATP, ADP, and UTP were selected as agonists as they are known to be agonists to one or more of the receptors expressed by our cells.

Primer name	Sequence	Product size (bases)
P2Y1 For	TCTACAGCATGTGCACGACCG	
P2Y1 Rev	GGCCCTCAAGTTCATCGTTTTC	212
P2Y2 For	CGAGAGGAGCCCCTTGTGGC	649(v1)
P2Y2 Rev	CCGTGCTGAAGGGCCAGTGG	515(v2,v3)
P2Y4 For	TCGTGCCCAACCTGTTCTTT	
P2Y4 Rev	TAAATGGTGCGGGTGATGTG	304
P2Y6 (I) For	CGCGGCGCAGATAACAAGACCTC	
P2Y6 Rev	AGGCGAAGTCGCCAAAGGGC	425
P2Y6 (II-IV) For	CACGGACTGCAAGCGAGGCA	450
P2Y11 For	AAAGGCACGCTGGCCTGCTC	
P2Y11 Rev	GGTCGCTGACTGCCAGCTGG	364
P2Y12 For	CCAGATGCCACTCTGCAGGTTGC	
P2Y12 Rev	CCTCATCGCCAGGCCATTTGTGA	235
P2Y13 For	ACTGCCGCCATAAGAAGACAGAGAG	
P2Y13 Rev	GGAGGAGCTGGGGATGTGAACA	225
P2Y14 (I) For	GAGCTGGGCTCCAAAGAATACTGGA	
P2Y14 Rev	CAGCTGCCAGGGACCAAGGC	637
P2Y14 (II) For	GCATGACTCTCACAGATGAAGGCC	432

Table 3.1: RT-PCR primer sequences for P2Y receptor subtypes. Courtesy of Jomnarong Lertsuwan.

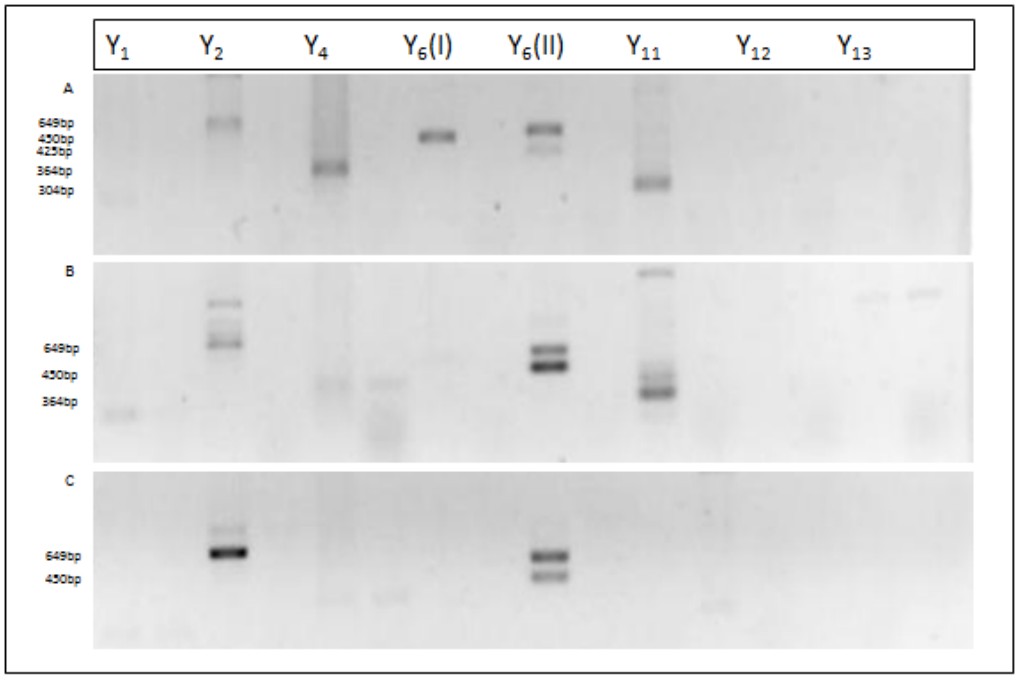
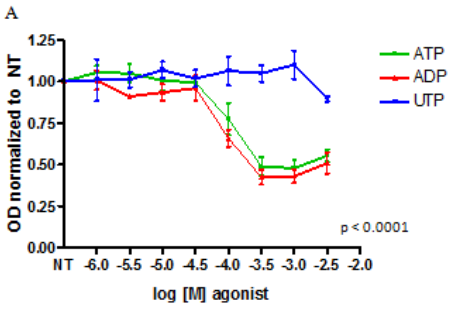
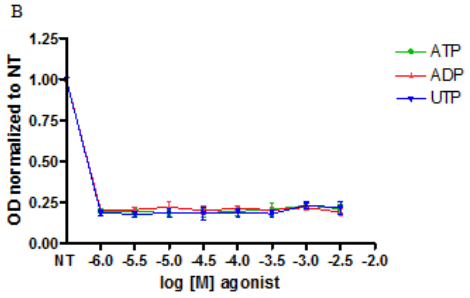


Figure 3.1: RNA expression of P2Y receptors in triple negative breast cancers. These representative images depict the RNA expression of P2Y receptor subtypes in (A) SUM149 cells, (B) GI101A cells, and (C) GILM2 cells. The SUM149 cells express the most receptor subtypes, including $P2Y_2$, Y_4, Y_6 (I and II represent different splice variants), and Y_{11}. G1101A show RNA expression of $P2Y_2$, Y_6, and Y_{11} while the isogenic progression of GI101A, the GILM2, show expression of only $P2Y_2$ and $P2Y_6$. $P2Y_{14}$ was not expressed in any of the three cell lines (data not shown).

SUM149, GI101A, and GILM2 cells were seeded at a density of 5000 cells per well using a 24- or 96-well plate. After allowing cells to attach overnight, the cells were treated daily with full log stocks ranging from 1µM to 1mM and half log stocks ranging from 3µM to 3mM for 7 days. Half media changes were performed prior to treatment each day. On day 8, 5mg/mL methylthazoltetrazolium (MTT) was added to each well to evaluate cellular proliferation. Figure 3.2 shows that SUM149 cell proliferation was affected greatly by treatment with ATP and ADP, with IC_{50} values of approximately 250µM and 300µM, respectively. UTP, however, had no effect on cell proliferation (panel A). These observations illustrate that adenine nucleotides have an abrogating affect on cell proliferation by an as yet unidentified means.

Conversely, in both GI101A cells and GILM2 cells, treatment with ATP, ADP, and UTP shows a drastic reduction in cell proliferation, with IC_{50} values <1µM (Fig. 3.2, panels B and C, respectively). It was suggested that these unexpected results may be due to the particular way in which the GI101A and GILM2 cells grow in 2D tissue culture dishes. Rather than forming a confluent monolayer like the SUM149 cells, these two cell lines organize themselves into "colony-like" formations as illustrated in Figure 3.3 that I believe affected the way in which the individual cells were exposed to the nucleotide agonists.





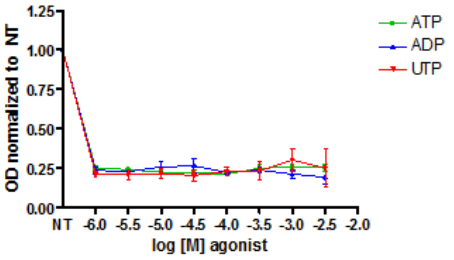


Figure 3.2: Breast cancer dose response assays. Cell proliferation measured by MTT assay. SUM149 (A), GI101A(B), and GILM2 (C) were treated with full and half log stocks of ATP, ADP, and UTP for 7 days. On day 8, 5mg/mL methylthazoltetrazolium (MTT) was added to each well and plates were incubated for 3h at 37°C. Following incubation, plates were read at 560nm on a PolarStar Optima plate reader. While the SUM149 cells demonstrate a significant dose response to ATP and ADP compared to non-treated cells (NT) ($p < 0.0001$ by linear regression), UTP had no effect on proliferation. IC_{50} of ATP ~250uM and ~300uM for ADP. The GI101A and GILM2 cells showed a drastic reduction in proliferation compared to non-treated cells (NT) with an IC_{50} of <1.0uM agonist.

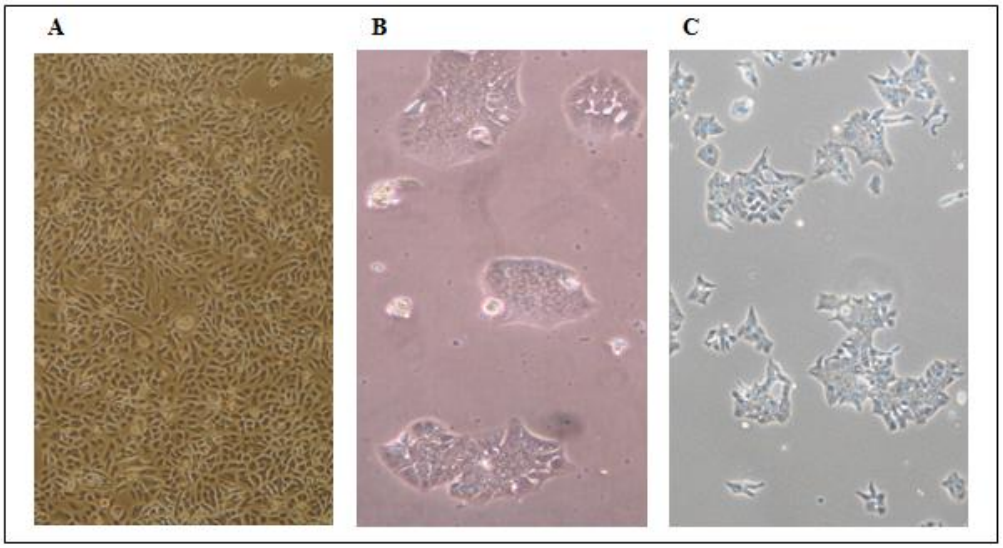


Figure 3.3: Triple negative breast cancer cells in monolayer. Representative images of the growth patterns of SUM149 (A), GI101A (B) and GILM2 (C) cells. Note the "colony-like" clusters of the GI101A and GILM2 cells. SUM149 image courtesy of Madhura Joglekar.

3.2.3 Focus Forming Ability of GI101A and GILM2 Cells Following Nucleotide Treatment with ATP, ADP, and UTP

Due to the particular way in which the GI101A and GILM2 cells grow in 2D, I chose to perform focus formation assays in order to determine if nucleotide treatment had any effect on cell proliferation. GI101A and GILM2 cells were seeded at a density of 5000 cells per well and allowed to attach overnight. Cells then were treated daily with 250μM of ATP, ADP, or UTP, performing half media changes prior to treatments. This concentration was chosen as it was close to the IC_{50} of ATP and ADP for the SUM149 cells. After 7 days of treatment, cells were trypsinized, counted, and replated at 1:500 and 1:1000 dilutions of cells to medium and allowed to grow for 10 days. Figure 3.4A and B shows that at both dilutions, GI101A cells were not affected by treatment with ATP, ADP, or UTP. However, GILM2 cells at a 1:500 dilution showed significant reduction in proliferation compared to non-treated cells (Figure 3.4C)(* $p < 0.05$ by paired students t-test). Figure 3.4D. shows that the 1:1000 dilution of GILM2 cells had a slight reduction in proliferation by ATP and ADP, however this was not significant. These results illustrate that GI101A cell proliferation is unaffected by nucleotide treatment, regardless of cell number. On the other hand, GILM2 cell proliferation by adenine nucleotides is reduced by an as yet unknown means.

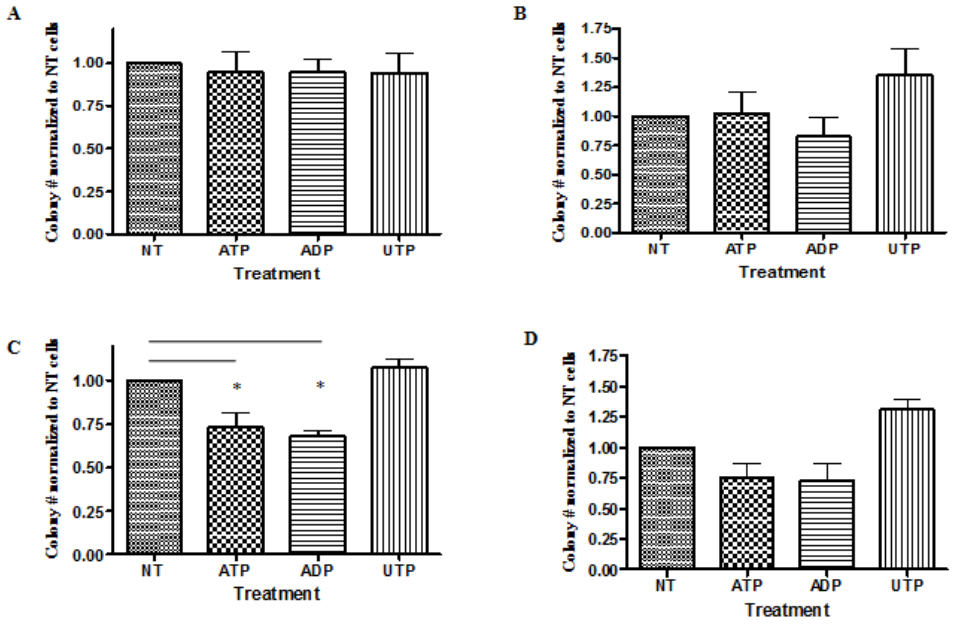


Figure 3.4: Focus formation assay of GI101A and GILM2 cells. Following daily treatment of cells with ATP, ADP, or UTP for 7 days, GI101A cells were plated at a dilution of 1:500 and 1:1000. GILM2 cells were plated at identical dilutions. After 10 days of growth, cells were stained with 0.4% crystal violet in 20% methanol for 1h at RT and colonies >10 cells were counted as such. The GI101A cells were unaffected by nucleotide treatment at either dilution (panels A and B). At a 1:500 dilution, GILM2 colony formation was significantly inhibited by ATP and ADP treatment compared to non-treated (NT) cells ($p < 0.05$ by paired students t-test [panel C]). A similar trend was observed at the 1:1000 dilution, however it was not significant (panel D). UTP has no effect on colony formation at either dilution.

3.2.4. The Effect of 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate and ATP + Apyrase on Cell Proliferation

As the effect of adenine nucleotides was shown to reduce proliferation of SUM149 and GILM2 cells, I chose to investigate whether a non-hydrolysable form of ATP, 2'(3')-O-(4-Benzoylbenzoyl)adenosine 5'-triphosphate (herein referred to as BzATP) would show similar results to those seen with ATP. Additionally, we used ATP combined with apyrase, an ATP diphosphohydrolase that removes the gamma phosphate from ATP and the beta phosphate from ADP, yielding adenosine monophospate (AMP). The three cell lines were treated as described previously. Figure 3.5A shows that the BzATP treatment had a similar effect as ATP on SUM149 cell proliferation but a higher dose was needed (IC_{50} approximately 2.25mM). However, proliferation was reduced to a greater extent at a total agonist concentration of 3mM. In Figure 3.5B, ATP + apyrase treatment indicates a modest effect on proliferation compared to non-treated cells, however it was not significant. The GI101A (Fig. 3.5C and 3.5D) and GILM2 (Fig 3.5E and 3.5F) cells, regardless of dilution, saw a significant reduction in proliferation when treated with BzATP. ATP + apyrase had no effect on proliferation in either cell line. These results suggest that the reduction in proliferation is most likely mediated through ATP and not AMP.

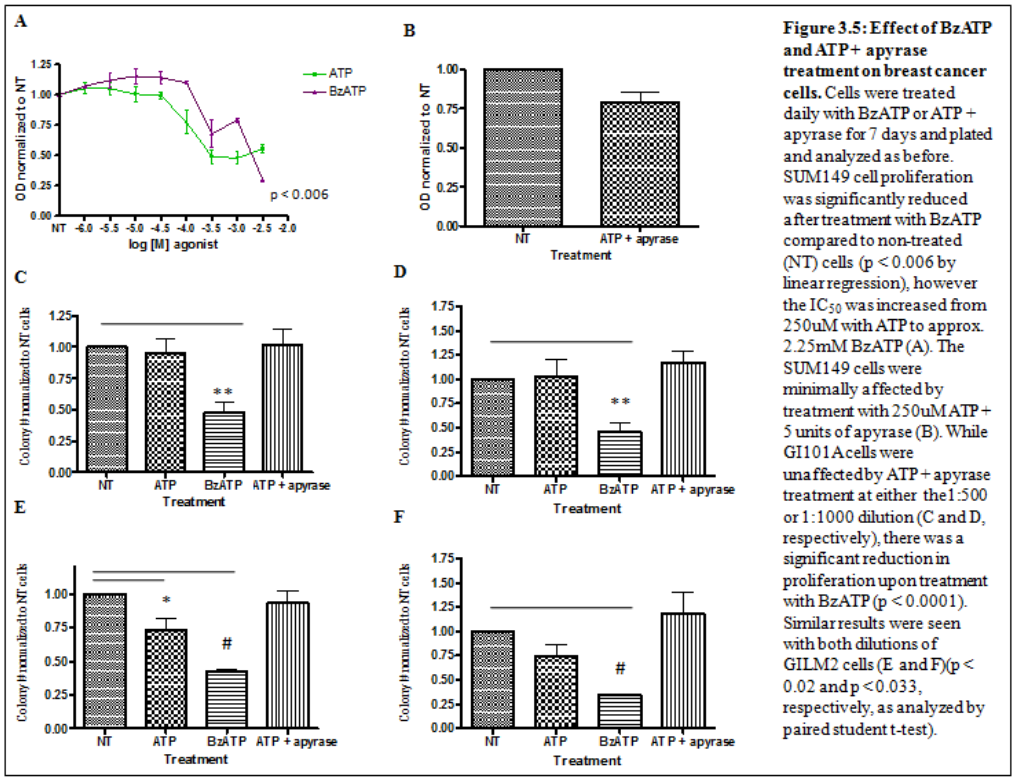


Figure 3.5: Effect of BzATP and ATP+ apyrase treatment on breast cancer cells. Cells were treated daily with BzATP or ATP + apyrase for 7 days and plated and analyzed as before. SUM149 cell proliferation was significantly reduced after treatment with BzATP compared to non-treated (NT) cells ($p < 0.006$ by linear regression), however the IC_{50} was increased from 250uM with ATP to approx. 2.25mM BzATP (A). The SUM149 cells were minimally affected by treatment with 250uM ATP + 5 units of apyrase (B). While GI101A cells were unaffected by ATP + apyrase treatment at either the 1:500 or 1:1000 dilution (C and D, respectively), there was a significant reduction in proliferation upon treatment with BzATP ($p < 0.0001$). Similar results were seen with both dilutions of GILM2 cells (E and F)($p < 0.02$ and $p < 0.033$, respectively, as analyzed by paired student t-test).

3.2.5. The Effect of ATP, ADP, and UTP Treatments on Triple Negative Breast Cancer Cell Invasion

The previous experiments demonstrate the impact of nucleotide treatment on the proliferation of triple negative breast cancer cells lines. We then chose to investigate whether these nucleotides may affect other areas of cell physiology. The nature of IBC and TN-nonIBC are such that they are highly lethal due to their invasive and metastatic abilities. Thus, we evaluated the effect of nucleotide treatment on cell invasion. Since treating the cells with 250μM of with certain nucleotides showed a reduction in cell proliferation, we decided to treat each cell line at a concentration of 250μM for this assay. Once the cells were seeded into the invasion well they were treated and and allowed to incubate for 24 h. The ability of the cells to invade through Matrigel relative to non-treated cells was determined using Volocity software. Figure 3.6 illustrates a trend towards increased invasion in the SUM149 cells upon treatment with ATP and UTP, while ADP appears to be less effective. Conversely, treatment of GI101A cells with UTP shows a trend towards decreased invasion. The role that ATP and ADP play in GI101A cell invasion is unclear due to fluctuations in data. When treating the GILM2 cells with UTP, trends toward decreased invasion were observed. In contrast, ATP and ADP show trends toward increased invasion when applied to these cells.

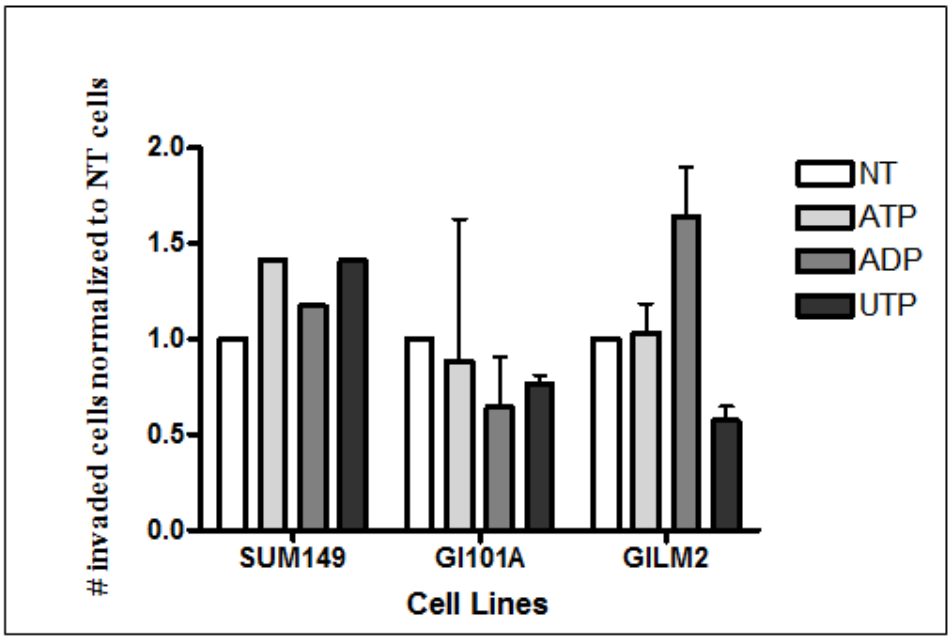


Fig 3.6: Triple negative breast cancer *in vitro* invasion assay. Breast cancer cells were plated into invasion wells and treated with 250uM nucleotide. After incubation for 24 h, the number of invaded cells was assessed using Volocity software. Values represent the number of invaded cells normalized to non-treated (NT) cells.

Chapter 4

THE EFFECT OF ADENOSINE ON TRIPLE NEGATIVE BREAST CANCER CELL PROLIFERATION

4.1 Introduction

In previous studies, I showed that triple negative breast cancer cell lines are differentially affected by treatment with nucleotides. ATP and its derivatives were shown to reduce proliferation of SUM149 and GILM2 cells; in GI101A cells I saw a decrease in invasiveness when cells were treated with these nucleotides. These data support other studies that show that ATP and other nucleotides, though specific P2Y receptor subtypes, are responsible for inhibition of cell proliferation (Katzur, 1999; Hopfner, 2001). Also, the data show that there may be an effect on cell invasion. Nevertheless, there is yet another aspect of purinergic signaling that may be involved in the development and progression of triple negative breast cancers. In this study, I sought to identify the adenosine receptor subtypes expressed in our chosen cell lines and analyzed the effect of adenosine treatment on proliferation of breast cancer cells.

4.2. Results

4.2.1 Adenosine Receptor Expression in Triple Negative Breast Cancer Cells

The literature indicates that there exist four adenosine receptor subtypes and that the expression of these subtypes can vary based on the specific tissue of interest and also among the cell types that comprise the tissue. Depending on the adenosine receptor subtype expressed, different signaling cascades may be initiated, ultimately affecting what cellular processes are activated. In this study, I sought to investigate the adenosine receptors present in each of the triple negative breast cancer cell lines. The cDNA for each cell line was used in the RT-PCR reaction with primers for each of the four subtypes. Three different sets of forward primers were used for the A_3 subtype to account for the different splice variants. The RT-PCR products then were separated in 1.5% agarose gel for 45 minutes at 100 volts. RT-PCR images (Fig 4.1 A-C) show the receptor subtypes expressed for each cell line. Contrary to what I expected, all three cell lines express only the A_1 receptor subtype.

Primer name	Sequence	Product size (bases)
A1 For	GTGGACCGCTACCTCCGGGT	
A1 Rev	TAC TTC TGC GGG TCG CCG GA	368
A2A For	TGTACATGTGAGCAGCCGCGT	
A2A Rev	GTCACCAAGCCATTGTACCGGAGCG	801
A2B For	CGAACTTTGGGCTCGGGCGAGTG	
A2B Rev	TGGCTGCATGGATCTCCCGCT	959
A3v1 For	TCTGCGCCATAGTGGGCAACG	
A3v1 Rev	GATAGAATGCACCCAGGGAGCCC	316
A3v2 For	GACTCTGCGCCATAGTGGGCA	
A3v2 Rev	CCAGGGCCAGCCATATTCTTCTGT	327
A3v3 For	GGGCTGCTTGAGTCCTGAGTCAC	
A3v3 Rev	CCAGTACCAGCCCGTGTCCTCT	376

Table 4.1: RT-PCR primer sequences for adenosine receptor subtypes. Courtesy of Jomnarong Lertsuwan.

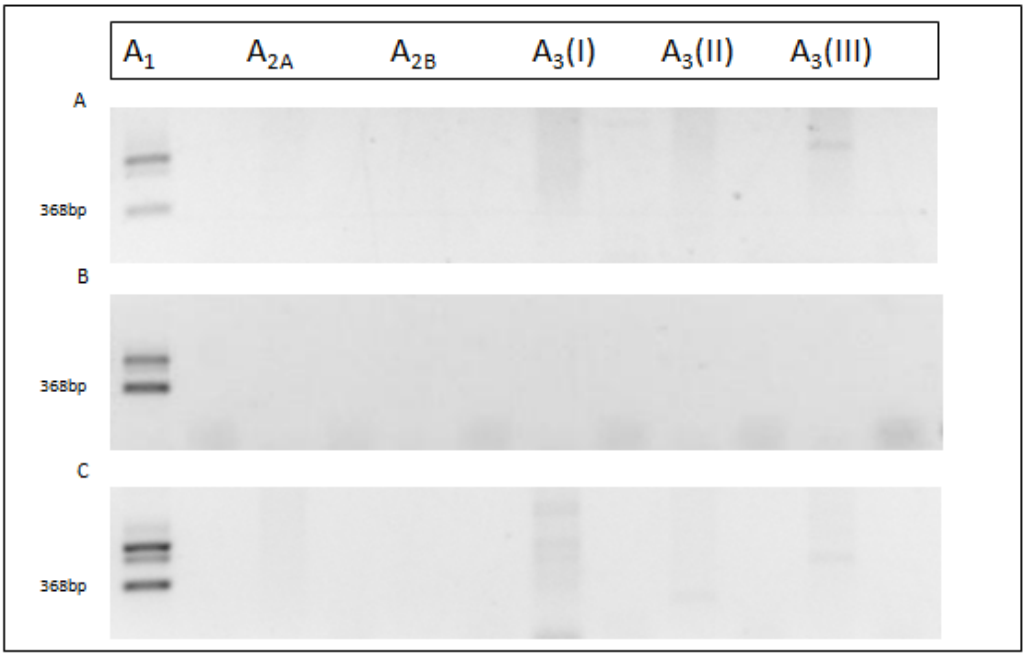


Figure 4.1: RNA expression of adenosine receptors in triple negative breast cancers. These representative images depict the RNA expression of adenosine receptor subtypes in (A) SUM149 cells, (B) GI101A cells, and (C) GILM2 cells. All three cell lines appear to express the mRNA of only the A_1 subtype

4.2.2 Adenosine and its Effect on Cell Proliferation

Having identified the possible receptor subtypes expressed on these cells, I performed a dose response assay on the triple negative IBC cell line, SUM149, in order to determine the effect of adenosine on cell proliferation. Cells were treated as described previously. Likewise, the triple negative non-IBC cell lines, GI101A and GILM2, were treated with 250μM adenosine in a focus formation assay as described previously. As shown in Figure 4.2A, when SUM149 cells were treated with adenosine, cell proliferation was significantly reduced. While the IC_{50} for adenosine was slightly increased compared to ATP (approximately 650μM vs. 250μM), overall it resulted in a greater inhibition of proliferation. Conversely, GI101A cell proliferation was unchanged from non-treated levels at either cell dilution (Fig. 4.2B and C). Similar results were seen at both dilutions of the GILM2 cells (Fig. 4.2D and E). Taken together, these results show that adenosine has a discernable impact on proliferation in IBC cells that is not seen in the non-IBC cells.

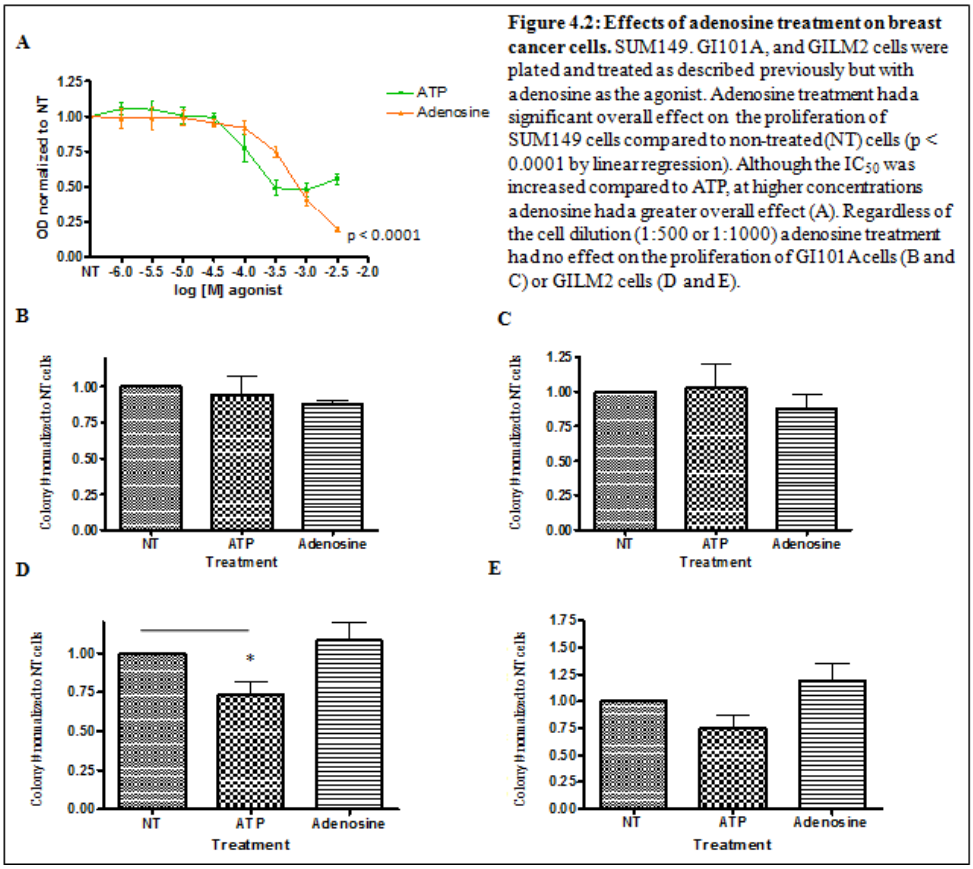


Figure 4.2: Effects of adenosine treatment on breast cancer cells. SUM149. GI101A, and GILM2 cells were plated and treated as described previously but with adenosine as the agonist. Adenosine treatment had a significant overall effect on the proliferation of SUM149 cells compared to non-treated (NT) cells ($p < 0.0001$ by linear regression). Although the IC_{50} was increased compared to ATP, at higher concentrations adenosine had a greater overall effect (A). Regardless of the cell dilution (1:500 or 1:1000) adenosine treatment had no effect on the proliferation of GI101A cells (B and C) or GILM2 cells (D and E).

Chapter 5

DISCUSSION

5.1. P2Y Receptor Expression in Triple Negative Breast Cancers and the Effect of P2Y Agonists on cell Proliferation and Invasion

The potential role for P2Y receptors in human cell physiology has been studied extensively since Burnstock first proposed the signaling actions of extracellular nucleotides in 1972. Numerous papers since have demonstrated the role of P2Y receptors in normal physiological as well as pathological states (Neary et al, 1999; Hopfner et al, 2001; Rumjahn et al, 2007; Bilbao et al, 2010). While some describe the P2Y expression profiles of virtually every tissue within the human body (as well as animal tissues), few of these discuss the receptors present in breast tissue (Buxton et al, 2010; White and Burnstock, 2006). My study is the first to examine the differences in expression among triple negative breast cancers: the inflammatory breast cancer (IBC) cell line SUM149 and the non-IBC cells GI101A and GILM2.

The RT-PCR results for each of the three cell lines supports my hypothesis that there exists a differential expression of the P2Y receptor subtypes between the TN-IBC cells and the non-IBC cells. SUM149 cells expressed the most P2Y subtypes, including $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ (refer to Figure 3.1). These subtypes are coupled

predominantly to the G protein subunit $\alpha_{q/11}$, which leads to signaling pathways responsible for cellular processes like growth, metabolism and proliferation (Abbracchio et al, 2006; Alberts et al, 2008). $P2Y_2$ and Y_6 also have been shown to couple to the G protein subunit $\alpha_{12/13}$, ultimately responsible for Rho activation and cytoskeletal rearrangements necessary for cell motility (Warzfeld et al, 2008). A third G protein that may be involved is $G\alpha_{15/16.}$ This protein has been shown to exist primarily in hematopoietic cells as well as aggressive cancers like IBC and pancreatic cancer (van Golen, 2011). It has been demonstrated that $G\alpha_{15/16}$ is capable of partially activating RhoC, a transforming oncogene shown to be expressed in various cancers and is believed to be a major contributor to the IBC phenotype (van Golen et al, 2000). Indeed, the hallmark of IBC is rapid onset and the formation of distant metastases early in the course of the disease as a result of tumor emboli forming and spreading through the lymphatics (Radunsky and van Golen, 2005). Further experiments need to be done to confirm the presence of $G\alpha_{15/16}$ in SUM149 cells; however, I believe that these P2Y receptor subtypes may be contributing to the development and progression of IBC by nucleotide signaling through the P2Y receptors, upregulating proliferation and cell motility.

The GI101A and GILM2 cells are representative cell lines of triple negative non-IBC breast cancer. While the 5-year disease-free survival rate is higher for patients diagnosed with triple negative breast cancer compared to those diagnosed with inflammatory breast cancer (68% vs. 35%), triple negative breast cancer is a

particularly aggressive disease with a high rate of metastasis and recurrence. In effort to better understand the metastatic process, the GI101A and GILM cell lines were developed in 1991 (Hurst et al 1991). The GILM2 cell line is an isogenic progression of the parental GI101A cells. GILM2 was developed from a lung metastasis in a xenograft mouse where the GI10A cells were injected into the mammary fat pad of the mouse. The resulting lung metastasis was excised and allowed to grow in a dish. These cells were subsequently named GILM1. The second cycle of *in vivo* selection yielded GILM2. It has been demonstrated that the *in vivo* selected cell lines were more aggressive (i.e. increased tumor development, increased proliferation) (Lev et al, 2003). The parental cell line, GI101A, showed RNA expression of the $P2Y_2$, Y_6, and Y_{11} subtypes while the GILM2 cells appear only to express RNA of the $P2Y_2$ and Y_6 subtypes. The expression levels of these receptor subtypes among these cell lines can be quantitatively determined by qRT-PCR and is an option for future experiments for this study. It may be that subtle differences in subtype expression (or expression levels) could explain the more aggressive and metastatic phenotype of GILM2 (versus the parental line GI101A). As in the SUM149 cells, it may be that the pathways responsible for proliferation and motility are being activated in the GI101A and GILM2 cells through the $P2Y_2$ and Y_6 subtypes, known to be primarily linked to the Gα protein subunit $_{q/11}$. Ultimately, these results indicate that the differential expression of P2Y receptor subtypes may explain the phenotypic differences between IBC and non-IBC.

ATP has been shown in numerous studies to decrease tumor growth in various malignancies, including colorectal (Höpfner et al, 2001), endometrial (Katzur et al, 1999), and breast (Abraham et al, 2003), although it remains unclear as to whether proliferation is impacted directly or whether these nucleotides have some sort of proapoptotic effect. My results indicated that treating SUM149 with the nucleotides ATP and ADP significantly reduced proliferation, with an IC_{50} between 250 and 300μM for both (Fig 3.2A). What was interesting to note was the lack of dose response to nucleotide treatment in the GI101A and GILM2 cells. A concentration of <1μM had a drastic effect on proliferation in these cell lines. This response was maintained despite increase in nucleotide concentration. I believe that this peculiar response may be, in part, due to the way in which these cell lines grow in 2D culture. The "colony-like" formations could be affecting how the nucleotides are distributed to the cells. A future study to examine this possibility is to perform dose response assays in a 3D culture system to see if the cell response changes. In order to study the effect of nucleotide treatment on these cells, it was suggested to me to try a focus formation assay (see materials and methods section for a description). As with the SUM149 cell line, I showed a significant reduction in cell proliferation in the 1:500 dilution of GILM2 cells at a concentration of 250μM of ATP and ADP. A similar trend was seen in the 1:1000 dilution of cells, however the results were not significant. Interestingly, the proliferation of GI101A cells was unaffected by treatment with ATP, ADP, or UTP at either concentration of cells (see Figure 3.2A and Figure 3.3). ATP and ADP

have been shown to be agonists of the $P2Y_2$ and Y_6 receptors, respectively (Abbracchio et al, 2006). Both receptor subtypes are expressed in these three cell lines. Thus, these results are supported by the evidence that ATP is responsible for the inhibition of growth and proapoptotic signals through $P2Y_2$ in colorectal carcinoma cells (Höpfner et al, 2001) and endometrial cancer cells (Katzur et al, 1999). However, other studies on breast cancer cells suggest that $P2Y_2$ and Y_4 expression stimulates cell proliferation in MCF-7 cells (Bilbao et al, 2010). It is still not clear why the activation of $P2Y_2$ in one cell line causes a reduction in proliferation while stimulating proliferation in others. The canonical signaling pathway from $P2Y_2$ activates phospholipase C (PLC) via $G\alpha_{q/11}$, ultimately leading to released calcium from cellular stores and transcriptional changes (Lodish, 2007). Perhaps the explanation of these conflicting data lies within the differences among cell types and the primary tumor from which they were derived.

To explain the inconsistent effectiveness of ATP between the IBC cell line, SUM149 and the non-IBC cells GI101A and GILM2, the differences in expression of P2Y receptor subtypes remains a possibility. Of the three cell lines, SUM149 is the only line that expressed $P2Y_4$. One of the primary antagonists of $P2Y_4$ is ATP (Abbracchio et al, 2006). Therefore, the phenotypic differences in proliferation in response to treatment with ATP, ADP, and UTP may be in part mediated by the presence of $P2Y_4$.

The observation that ATP, ADP, and UTP had no effect on the GI101A cells was interesting. A possible explanation for this may be the presence of ectonucleotidases on the surface of these cells. Ectonucleotidases are enzymes that break down the triphosphate forms of nucleotides into their di- and monophosphate forms (White and Burnstock, 2006). E-NTPDases (ecto-nucleotide triphosphate diphosphohydrolase) and E-NPPases (ecto-nucleotide pyrophaphatase/phosphodiesterase) are two such enzymes (Zimmerman, 2000). Another possibility is the presence of ectoenzymes, e.g. alkaline phosphatase or acid phosphatase. In fact, the activity of acid phosphatase has been demonstrated in breast tissue. Histochemical detection has indicated that acid phosphatase is expressed at very low levels in normal breast tissue, yet is markedly elevated in breast cancer tissue (Halaby et al, 2001). The role of acid phosphatase in altering the availability of extracellular nucleotides seems plausible when considering the tumor environment. The high rate of metabolism in and around the tumor can shift the pH of the local cell environment downward, thus allowing these phospatases to function more affectively.

The GI101A cells were demonstrated to express $P2Y_2$, Y_6 and Y_{11} RNA. The agonists for these receptors include ATP, UTP and/or ADP. It is possible that the GI101A cells express levels of these ectonucleotidases/ectoenzymes that prevent the actions of ATP, ADP, and UTP – at least at the concentrations at which they were applied. This might explain why the SUM149 and GILM2 cells - both of which have

common P2Y receptor subtype expression with GI101A – responded to nucleotide treatment and the GI101A cells did not.

Based on the results of the previous experiment, I next investigated whether a non-hydrolysable form of ATP, known as BzATP, would show similar effects to those seen with ATP. Additionally, I treated cells with ATP combined with apyrase to see if the cells would respond. Apyrase is an enzyme that cleaves the gamma and beta phosphates from ATP to form AMP. The results of the experiment indicated that BzATP significantly reduced cell proliferation in SUM149, GI101A and GILM2 cells. In the case of the GI101A cells and GILM2 cells, BzATP was more effective than ATP in reducing proliferation. The use of derivative ATP agonists on tumor cells has been demonstrated in bladder cancer cells (Shabbir et al, 2007). This study showed that ATP and BzATP were equally effective in reducing proliferation of bladder cancer cells *in vitro*. In fact, ATPγS (another non-hydrolysable form of ATP) demonstrated the greatest reduction in cell viability compared to other analogs of ATP. The use of BzATP prevented the cleavage of phosphate groups from the molecule, thus allowing me to determine whether the observed actions of ATP previously seen are mediated by ATP or by another molecule. It was interesting to note that while ATP was ineffective in regard to the GI101A cells, BzATP significantly reduced their proliferation compared to untreated cells. This supports the supposition that ectonucleotidases/ectoenzymes may be present on these cells and

could be interfering with the availability of nucleotides at the P2Y receptor binding site.

In contrast with the considerable effect of BzATP, ATP + apyrase treatment showed only a modest reduction of proliferation in SUM149 cells compared to untreated cells (Figure 3.5A). The GI101A and GILM2 cells were virtually unaffected by ATP + apyrase treatment. The enzymatic actions of apyrase ultimately produce AMP. The results from this experiment indicated that AMP does not appear to contribute much, if at all, to the decrease in cell proliferation seen with treatments using ATP or ADP. Despite the modest impact on proliferation overall, the results indicated that there was a difference in response between the IBC and non-IBC cells. The disparity between the responses of these cells to nucleotides may again be explained by ectonucleotidases/ectoenzymes. Further breakdown of AMP by alkaline phosphatase (AP) yields adenosine, a molecule that has been shown to reduce proliferation in several cancers, including colonic adenocarcinoma (Lelièvre et al, 1998) and prostate cancer (Aghaei et al, 2012). It may be that the effects of ATP + apyrase on SUM149 proliferation are due to the conversion of AMP to adenosine by specific ectonucleotidases like AP that may not be expressed in GI101A and GILM2 cells. I will discuss the possible role of adenosine on cell proliferation later.

My previous experiments examined the role of ATP, ADP, and UTP on triple negative breast cancer cell proliferation. I then asked if these nucleotides may play a role in cell invasion. The hallmark of inflammatory breast cancer is the ability of the

tumor cells to invade into the dermal lymphatics of the breast as a means to metastasize to the visceral organs, including the liver and lungs (Radunsky and van Golen, 2005). Triple negative breast cancers, while not as lethal as IBC, are another type of less common breast cancers that are particularly aggressive and metastatic. Thus, I wondered if there may be a difference in invasiveness between the IBC cell line, SUM149, and the triple negative non-IBC cell lines GI101A and GILM2 when treated with ATP, ADP, and UTP. The results of these experiments indicate that the cell lines did indeed respond differentially to the nucleotide treatments. SUM149 invasion may be stimulated by all three nucleotides, but particularly by ATP and UTP. GI101A, on the other hand, shows a potential reduction in invasiveness when treated with ADP and UTP. The GILM2 line, derived from the GI101A, showed an increase in invasion when treated with ADP and a decrease when treated with UTP (see Figure 3.6). These data support our previous observations seen in the cell proliferation assays. The SUM149 and GILM2 cells showed reduced proliferation when treated with ATP and ADP. Taken together, these data maintain the notion that invading cells are not proliferating. The GI101A cells, however, were unaffected by ATP, ADP, and UTP in the cell proliferation assays. In this study, we saw that UTP (and possibly ADP) negatively affected the invasive capability of the cells. Few papers discuss the role of P2Y receptor signaling in tumor cell invasion; however, it has been demonstrated that P2Y receptor activation via ATP was responsible for increased invasion of prostate

cancer cells (Chen et al, 2004). Additional studies that include other nucleotide derivatives may help to clarify the involvement of P2Y agonists in cell invasion.

5.2 The Effect of Adenosine on Triple Negative Breast Cancer Cell Proliferation

Adenosine is produced endogenously through the metabolism of ATP, ADP, and AMP that occurs in almost every cell of the body. Extracellular levels drastically increase during cell stress, e.g. hypoxia (Gessi et al, 2011). Hypoxic environments are characteristic of solid tumors of a certain size, thus explaining the high levels of adenosine found within the tumor microenvironment (Merighi et al, 2003). The cellular responses to adenosine, both in normal and cancerous tissue, depend on the adenosine receptor subtype(s) expressed within the tissue. The four subtypes are all G-protein coupled receptors, yet the heterotrimeric G proteins with which they are associated can vary based on the tissue type (Fredholm et al, 2012).

Adenosine has been shown to aid tumor cells in immunoevasion, preventing recognition of the cancer cells by the host immune cells, thereby allowing the cancer to progress unchecked. Specifically, A_{2A} ligands were shown to suppress the cytotoxic effects of activated killer cells (Gessi et al, 2011). In another study, CHO cells expressing A_1 receptors demonstrated an activation of ERK1/2 "at physiologically relevant concentrations" (Merighi et al, 2003). ERK1/2 is a key molecule in the MAPK pathway that canonically leads to increased cell proliferation (Lodish, 2007).

As mentioned previously, based on the results of my experiments on the role of UTP, ATP, and its derivatives on triple negative breast cancer cell proliferation, I wanted to investigate whether adenosine had any effect. Thus, I performed RT-PCR to evaluate the RNA expression profiles of adenosine receptor subtypes in these cell lines. Surprisingly, all three appeared express only the A_1 receptor subtype RNA (Figure 4.1). I then hypothesized that despite the identical expression profiles there may be differences in response to adenosine treatment. The results of the experiment indicated that adenosine treatment significantly reduced SUM149 cell proliferation, with an IC_{50} of approximately 1mM (Fig 4.2A). While this concentration is greater than the IC_{50} seen with ATP and ADP, overall adenosine had a greater impact on proliferation. It was interesting to note that the non-IBC cell lines were unaffected by adenosine treatment, regardless of the number of cells plated (Fig 4.2B-E). I believe that this differential response of the cells may in some ways explain the phenotypic differences between IBC and non-IBC. It is interesting to note that the A_1 adenosine subtype has been reported to have possible antiproliferative effects in colon, breast, and glioblastoma cells, which coincides with what I observed with the SUM149 cells upon adenosine treatment (Gessi et al, 2011). On the other hand, there exist various studies that report the opposite – A_1 receptor has a protumoral effect. For example, increased chemotaxis was observed in melanoma cells treated with A_1 ligands; MDA-MB-468 breast carcinoma cells showed an increase in cell cycle progression and proliferation (Gessi et al, 2011). The conflicting data from these studies could be

explained by differences in expression *level* of adenosine receptor subtypes, thus impacting the signal received by the cell. In fact, it has been reported that cancerous breast tissues express higher levels of the A_1 receptor subtype than in matched normal breast tissue (Gessi et al, 2011). Perhaps a variation in expression level also exists among the different forms of breast cancer, explaining the difference in response to adenosine seen here.

5.3 Significance

The experiments performed in this study hopefully will aid in understanding a bit more the intricacies of triple negative breast cancers. The differential effects observed among the cell lines and particularly between the IBC cell line and the non-IBC cell lines will provide insight into the role of adenosine and related nucleotides in the onset and progression of these devastating forms of breast cancer. The use of ATP in the treatment of cancer has been already demonstrated in the past. With the aid of additional knowledge, perhaps a new therapeutic strategy that combines the antiproliferative effects of ATP and its derivatives with current adjuvant therapy may be developed.

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APPENDIX

American Cancer Society

February 23, 2012

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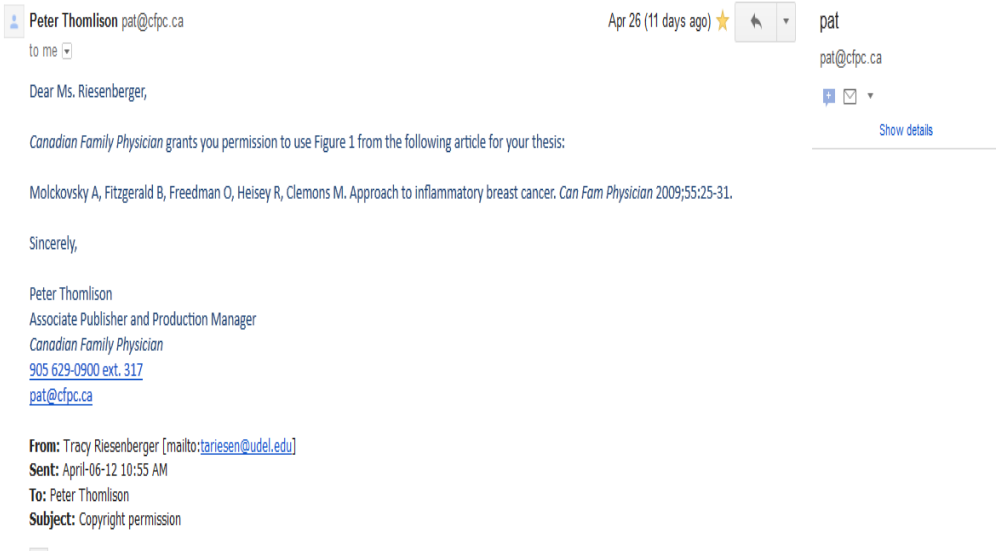

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Elizabeth Sandler esandler@aaas.org Feb 10

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