Neurotransmitter Signal Transduction and its Role in Pulmonary Arterial Hypertension Vasodilatation and Vasoconstriction

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degrees of Bachelor of Science

in

Biology and Biotechnology

and Biochemistry

by

__________________________  __________________________
Felipe Strefling              Jenny Strefling

April 29, 2010

APPROVED:

__________________________  __________________________
Alice Gardner, Ph.D.           David Adams, Ph.D.
Department of Pharmaceutical Science Biology and Biotechnology
Massachusetts College of Pharmacy WPI Project Advisor
Major Advisor
ABSTRACT

Pulmonary Arterial Hypertension (PAH) is a devastating disease characterized by a persistent increase in pulmonary arterial resistance. Endothelin is a hormone involved in maintaining vasoconstriction, but the enzymes and proteins involved in its signaling pathway are not fully known. Understanding the endothelin-B (ET\textsubscript{B}) signaling pathway is crucial for the development of a more specific endothelin-targeted therapy for PAH. Therefore, the objective of this project was to identify mediators utilized by the ET\textsubscript{B} receptor and help determine their function in the regulation of ATP neurotransmitter release. In rat PC12 cells differentiated by neuronal growth factor, the inhibition of PLC was shown to reduce ET\textsubscript{B}-mediated ATP release.
TABLE OF CONTENTS

Signature Page ........................................................................................................... 1
Abstract ...................................................................................................................... 2
Table of Contents ....................................................................................................... 3
Acknowledgements ................................................................................................... 4
Background ................................................................................................................ 5
Project Purpose ......................................................................................................... 20
Methods .................................................................................................................... 21
Results ....................................................................................................................... 28
Discussion .................................................................................................................. 33
Bibliography .............................................................................................................. 36
ACKNOWLEDGEMENTS

This project would not have been possible without the support of our advisor Professor Alice Gardner at the Massachusetts College of Pharmacy and Life Science (Worcester campus). Dr. Gardner provided us with the necessary background and skills to complete this project. We would also like to extend thanks to the faculty and staff of the pharmaceutical sciences department for allowing us to use laboratory resource and space. We would like to additionally thank Professor David Adams, our WPI advisor, for helping write this report and for technical advice.
Pulmonary Arterial Hypertension

Pulmonary Arterial Hypertension (PAH) is a progressive, symptomatic, and ultimately a fatal disorder that elevates the blood pressure in the pulmonary arteries, putting the patients’ median life expectancy at only 2.8 years (McGoon & Garvan, 2009). Although treatment for the disease has improved in the last decade, our limited understanding of the disease pathogenesis impedes the achievement of optimal outcomes. The pathobiology of PAH is composed of many factors, including increased pressure in the arteries that connect the lung to the heart. As a result, the right side of the heart has to work harder to pump blood through the lungs. As the disease progresses, morphological changes to the pulmonary vessel wall and coagulation increase pulmonary vascular resistance (McGoon & Garvan, 2009).

PAH was first described over 100 years ago in a patient with right-heart failure that was diagnosed with syphilitic pulmonary arteritis. Clinically PAH is defined simply as raised blood pressure in the pulmonary arteries. Normal pressure ranges from 15 to 25 mm Hg systolic, and 8 to 15 mm Hg diastolic, with mean pressure between 10 and 20 mm Hg. When mean pulmonary artery pressure increases to 25 mm Hg or higher, with a pulmonary capillary wedge pressure of 15 mm Hg or less, then the patient is diagnosed with PAH (Gaine & Rubin, 1998).

Pathophysiology of PAH

PAH, regardless of the cause, leads to the enlargement of the right ventricle of the heart as it attempts to compensate for abnormally high pressure in the pulmonary arteries. If the right ventricle cannot enlarge, then the pressure will increase in the right atrium and systemic venous system. Eventually symptoms of right-sided heart failure, or cor pulmonale, are manifested in
the patient. Patients often complain about fatigue, this is because the right ventricle cannot pump enough blood, which is deoxygenated, into the lungs to be oxygenated. The bone marrow attempts to compensate for the lack of oxygen by stepping up red blood cell production. Unfortunately this leads to polycythemia, a thickening of the blood. This puts even more stress on the heart since viscous blood is harder to pump, and clotting is more likely (Holcomb, 2005).

Signs and symptoms associated with PAH include reduced oxygenation, decreased cardiac output, and an inability to increase cardiac output, which leads to symptoms mimicking heart failure, such as shortness of breath, fatigue, and syncope (fainting). During increased activity these symptoms may increase and lead to angina-like chest pains. Direct signs of right ventricular failure include peripheral edema, hepatomegaly, tricuspid regurgitation, an S3 heart sound, prominent right ventricular impulse and jugular vein distension. Diagnosis is usually reached by analyzing a patient's medical history for congenital heart disease or the use of banned weight-reduction medication (Holcomb, 2005).

**PAH Classification**

Patients with PAH are divided into 4 classes of degree of severity. Class I patients have no limitation of physical activity, and ordinary activity does not cause fatigue, chest pain, or near syncope. Class II patients have slight limitation of physical activity. Patients are comfortable at rest, but ordinary physical activity causes fatigue, chest pain, or near syncope. Class III patients have limitations to physical activity. These patients are comfortable at rest, but less-than-ordinary physical activity causes fatigue, chest pain, and near syncope. Class IV patients cannot do any physical activity without symptoms. When at rest, these patients may be fatigued, and any
physical activity leads to discomfort. These patients show signs of right-sided heart failure (Cipla Doc, 2000).

In July of 2004, the American College of Chest Physicians reclassified PAH on the basis of etiology. Primary PAH became idiopathic PAH, or familial PAH if the cause is supported genetically. Idiopathic PAH occurs predominantly among young adults who are probably predisposed to the disorder. Other classifications are related to specific etiologies. PAH cases caused by underlying disease such as HIV or toxicity are much more common. Prior to widespread use of the weight-reduction drugs fenfluramine and dexfenfluramine in 1967, PAH was a relatively rare disease afflicting only 1-2 cases per million people annually. The rate of prevalence rose to 25-50 per million annually following the widespread use of these appetite suppressants. Although these drugs were banned from the market in 1997, PAH caused by these toxic drugs did not go away because of an illegal black-market. Also because of the prevalence of HIV the incidence rate of PAH has not been reduced (Holcomb, 2005).

**Cellular Pathways of PAH**

Various cellular pathway abnormalities are associated with the development and progression of PAH. Regardless of the cause all PAH patients have abnormal pulmonary endothelium function. The pulmonary endothelium's purpose is to maintain low pulmonary vascular resistance. In order to perform its job correctly, a balanced production of vasodilators (in this case prostacyclin and nitric oxide) and vasoconstrictors (such as endothelin-1, thromboxan A2, and serotonin) must be maintained. Therefore, patients with PAH are characterized with a decreased concentration of prostacyclin and nitric oxide, while the
production of thromboxan A<sub>2</sub> and endothelin-1 increases (Yildiz, 2009, p. 9). An increasing concentration of endothelin is associated with the progression of PAH.

However, endothelial cell dysfunction is just one of the causes of the disease. PAH can also be caused by other factors that lead to dysfunction of pulmonary circulation, such as activation of adventitial fibroblasts, or the alteration of extracellular matrix components. Factors that trigger the initiation of the disease are unknown, but once the disease is triggered the consequences include vasoconstriction, vascular smooth muscle cell and endothelial cell proliferation, and remodeling and thrombosis that cause the blood vessel to become narrower. As the disease progress, more collagen and smooth muscle cells are synthesized in the pulmonary artery in response to pulmonary artery pressure experienced by endothelial cells (Yildiz, 2009, p. 10). These changes in the vascular structure of the pulmonary artery lead to loss of function and eventually right ventricular dysfunction.

In 2000, a gene on chromosome 2 that encodes for bone morphogenetic protein receptor II (BMPR2) was found to be associated with familial pulmonary arterial hypertension (FPAH). Mutations in BMPR2 occur in 50% of patients with familial PAH, while 25% of patients with idiopathic PAH display this mutation (Holcomb, 2005). However, fewer than 20% of the individuals carrying the mutated version of this gene develop FPAH. About 65% of families with familial PAH have exonic or intronic allelic variants in the BMPR2 gene, and they are transmitted in an autosomal dominant manner. Also some 10% of sporadic cases of IPAH involve isolated exonic allelic variants of the BMPR2 gene (McGoon & Garvan, 2009, p. 1). The reason why BMPR2 mutates is still unknown, but researchers believe environmental stimuli or other genes interfering with BMPR2 may lead to its mutation (Gaine & Rubin, 1998, p. 2). The mutated allelic variants of BMPR2 cause amino acid changes in the BMPR2 protein that
interrupt signal transduction during the process of pulmonary vascular smooth muscle cell apoptosis, thus promoting cellular proliferation (McGoon & Garvan, 2009).

**PAH Treatments**

Treating PAH starts with removing any stressors or drugs that give rise to or exacerbate the disease. For example, the weight loss drugs fenfluramine and dexfenfluramine are known to cause PAH, so if a patient was taking one of these they are discontinued. PAH is not curable, so treatment is aimed at reducing pressure, removing excess fluid, and reducing the risk of clotting. The usual drug cocktail consists of vasodilators, anticoagulants, and careful use of diuretics. Supplemental oxygen is also advisable if blood oxygen saturation reaches below 90%. Patients with sleep apnea and PAH should use a Continuous Positive Airway Pressure device, as this treatment decreases pulmonary artery pressure. Modern pharmaceutical treatment for PAH includes calcium channel blockers, prostacyclin analogues, endothelin receptor antagonists, phosphodiesterase inhibitors, and thromboxane inhibitors, all of which dilate the pulmonary artery, and thus may stop the progression of PAH by maintaining endothelial integrity (Holcomb, 2005).

The first vasodilators used to treat PAH were calcium channel blockers. This approach only works with about 20% of patients with idiopathic PAH, and does not seem to help other types of PAH. Also patients with *cor pulmonale* must avoid calcium channel blockers because they decrease myocardial contractility and may thus induce heart failure. High doses of dihydropyridine calcium channel blockers are required for adequate dilation of the pulmonary arteries (Robbins, 2006). Initial administration must be done in a clinical setting to monitor for adverse reactions. An acute vasoreactivity test is recommended for calcium channel blocker
candidates. This test uses known short-acting vasodilators such as intravenous adenosine or epoprostenol or inhaled nitric oxide. About 25% of patients with PAH have a positive response to these short acting drugs, which is defined as a decrease in pulmonary arterial pressure of 10 to 40 mm Hg, and an increased or unchanged cardiac output (Mehta, 2003). Those with a negative response usually have a worse prognosis. Another side affect of calcium channel blockers is edema in the lower extremities, although this is usually controlled by diuretics (Rich, 2000, p. 3).

Only 30% of patients lived past 3 years after diagnosis before the development of vasodilators and endothelin receptor antagonists. These new drugs dilate the pulmonary arteries, reducing pressure and thus increasing life expectancy. Epoprostenol, a prostacyclin approved by the FDA in 1995, is administered via continuous I.V. infusion through a long-term central venous access device by a portable battery operated pump. This drug is a candidate for Class III and IV patients who are not candidates for calcium channel blocker therapy. Treprostinil, an analogue of prostacyclin, is given as a continuous subcutaneous infusion. It was approved in 2002 and has boosted the survival rates to over 65%. Again the guidelines stipulate that this drug should only be given to Class III and Class IV patients that are not candidates for calcium channel blockers. Iloprost is a newer drug similar to prostacyclin that is inhaled, avoiding all the risks associated with a continuously administered injectable. It is only given along the same guidelines as Epoprostenol (Holcomb, 2005).

In 2001, Bosentan, an endothelin receptor antagonist, became the first oral drug for PAH approved by the FDA. Only used for Class III and IV patients, this drug may stop progression of PAH or may even reverse it. Future research focuses on finding better pulmonary vasodilators that have a convenient admission route while also not causing systemic hypotension (Holcomb, 2005).
**Endothelin**

An increasing concentration of endothelin is associated with the progression of PAH. As PAH progresses, changes in the vascular structure become apparent, such as inflammation, vasoconstriction, cell proliferation, hypertrophy, and the formation of plexiform lesions. These changes in the vascular structure of the pulmonary artery lead to loss of function and eventually right ventricular dysfunction (Goraca, 2002). Hickey discovered an unknown factor produced in the endothelium that causes smooth muscles to contract (Galie et al., 2004). By 1988, Yanagisawa isolated and identified this factor from pig arterial endothelial cells, and called it endothelin-1 (ET-1) (Galie et al., 2004).

Endothelin-1 is a 21 amino acid peptide with a molecular weight of 2492 daltons (Figure 1). There are currently 3 known isomers (ET-1, ET-2, and ET-3). Endothelin has two disulfide bonds, one between cysteine amino acids 1 and 15, and another between 3 and 11. The presence of these disulfide bonds is critical because it contributes to the biological activity of ET (Goraca, 2002). All three isoforms share a common structure that is characterized by the existence of two disulfide bridges, a loop configuration, and an active site at the C terminus according to figure 1 (Bouallegue & Srivastava, 2007).
Figure 1: Diagram of Endothelin Primary Structure and the Factors Controlling its Regulation (Piuhola, 2002).

Many mammalian species, including humans, carry genes encoding endothelin. Three separate genes encode ET-1, ET-2, and ET-3, but the resultant proteins are all made up of 21 amino acids with two disulfide bonds. The isomers ET-1 and ET-3 differ in 6 out of the 21 amino acid positions. This causes each endothelin variant to have different affinities toward different receptors. For example ET-1 has a higher affinity for the receptor ET_A compared to ET_B. All endothelins have equal affinity for the ET_B receptor. ET-1 is mostly expressed in endothelial cells, but it is also found in vascular and airway smooth muscle cells, leukocytes, marcophages, cardiomyocytes, and mesangial cells. ET-2 on the other hand is mostly found in the kidney and intestine. ET-3 is mostly expressed in the brain. This differing level of expression for each endothelin and its receptor is the cause behind the different regulatory mechanisms of each organ or tissue system (Miyauchi & Masaki, 1999).
There are many factors involved in the up regulation or down regulation of endothelin, among them, shear stress, extreme pH, oxidized low-density lipoprotein, glucose, insulin, angiotensin II, catecholamines, growth factors, nitric oxide, and prostacyclin (Ergul, 2002).

An elevated level of ET-1 is seen in patients with acute myocardial infarction, hypertension, and heart failure. Because of these observations, ET-1 has become associated with the pathophysiology of these disease states. ET-1 mediates its effects through two distinct heptahelical G-protein-coupled receptors, the endothelin ET_A and endothelin ET_B receptors (Kanai & Hasegawa, 2004). On the smooth muscles of the vascular bed, both receptors are found and both are responsible for mediating contraction. On the other hand, the endothelin ET_B receptors on endothelial cells are responsible for mediating vasodilatative factors such as nitric oxide (NO) and prostacyclin (Masaki et al., 1999).

Developmental studies of endothelin-deficient and endothelin-receptor-deficient mice have shown the important role of endothelin during embryonic development. ET-1-deficient mice soon die after birth because of abnormal development of craniofacial and cardiac muscles (Miyauchi & Masaki, 1999).

**Endothelin Signal Transduction**

Three signaling pathways are disturbed in patients with PAH: the nitric oxide pathway, the prostacyclin pathway, and the endothelin pathway (see figure 2). We will mainly focus on the endothelin pathway (Figure-2), since it is the major participant in the pathogenesis of PAH (McGoon & Garvan, 2009, p. 192).
Both endothelin ET$_A$ and ET$_B$ receptors bind to G-proteins on the cytoplasmic side of the membrane. These G-proteins are then linked to adenylyl cyclase which dephosphorylates ATP into cyclic AMP (cAMP). According to figure 2 $G_s$-protein (stimulatory G-protein) binds to the ET$_A$ receptor, and $G_i$-protein (inhibitory G-protein) binds to the ET$_B$ receptor. The activation of ET$_A$ thus releases cAMP, which then triggers the formation of PK-A (cAMP stimulated protein kinase), and eventually leads to the influx of calcium ion by phosphorylation of L-type calcium channels. If contraction persists, calcium ions from inside the cells are also released (Klabunde, 2007).
In figure 2, both endothelin ET\textsubscript{A} and ET\textsubscript{B} receptors are also linked to G\textsubscript{q}. This pathway triggers the release of intracellular and extracellular Ca\textsuperscript{++} by the activation of phospholipase C (PLC). Activation of PLC stimulates the formation of inositol triphosphate (IP\textsubscript{3}) from phosphatidylinositol biphosphate (PIP\textsubscript{2}). This increase in IP\textsubscript{3} triggers the sarcoplasmic reticulum in the heart to release calcium ions. However, under normal physiological conditions, voltage-operating calcium channels do not contribute much to cause muscle contraction. Instead, contraction is mediated by the activation of non-selective cation channels. When ET-1 level is high, this causes the intracellular free calcium ions to increase. In order to physiologically meet the high vasoconstriction necessary of PAH, extracellular calcium ions are released to enter the cell (Masaki et al., 1999).

Nitric oxide inhibits contraction by decreasing calcium entry through the non-selective cation channel. It is also used to inhibit the intracellular free calcium ion concentration triggered by ET-1 binding to its receptors. In guinea pig tracheal smooth muscle, the use of nifedipine (a voltage-operated calcium channel inhibitor) effectively inhibits ET\textsubscript{B} mediated contraction but not ET\textsubscript{A} mediated contraction. This suggests that there may be another molecular mechanism that induces contraction via the ET\textsubscript{B} receptor (Masaki et al., 1999).

**Co-Transmission in the Sympathetic Nervous System**

The sympathetic nervous system innervates many organs and tissues, one of them being the vasculature. Larger pulmonary arteries appear to be more richly innervated compared to smaller ones (Downing & Lee, 1980). These sympathetic innervations normally control the tone and thus pressure of the pulmonary arteries. For example, externally stimulating the upper thoracic sympathetic chain, middle cervical ganglia or thoracic vagosympathetic branches with a
current consistently raises pulmonary arterial pressure (10-15% over resting pressure) while maintaining constant blood flow. However, when the pulmonary arterial pressure is kept constant and flow is allowed to vary, stimulating the same nervous structures elicits a drop in blood flow by as much as 30% (Downing & Lee, 1980). Pulmonary arterial pressure is controlled by sympathetic neuronal release of neurotransmitters at the vascular neuroeffector junction. Specifically, the tone of the arteries is controlled by the noradrenergic neurons, which release neurotransmitters towards vascular smooth muscles. For example, arterial tone is maintained upon continuous stimulation of noradrenergic neurons releasing norepinephrine (NE). Increasing sympathetic stimulation of noradrenergic neurons results in arterial constriction. On the other hand, decreasing sympathetic stimulation of noradrenergic neurons results in arterial dilation (Craig, 2004). Increased arterial constriction, and thus resistance, is a sign of pulmonary arterial hypertension (Pulmonary Arterial Hypertension, 2005).

In the past, it was thought that postganglionic neurons used only one neurotransmitter. However, this has been proven incorrect by evidence obtained from anatomical and pharmacological studies. It is has now been demonstrated that multiple neurotransmitters are released by postganglionic neurons at the neuroeffector junctions. Neuropeptides Y, NE, or ATP were found upon sympathetic nerve stimulation. Histochemical studies have shown that immunoreactive neuropeptides are co-localized with NE, indicating that multiple neurotransmitters are released by the sympathetic postganglionic neurons. Additionally, neurotransmitters released as a result of sympathetic nervous system stimulation can function as co-transmitters, modulators, or co-mediators along with NE (Loewy & Spyer, 1990).

NE, ATP and NPY are the three main neurotransmitters/ neuromodulators released by postganglionic sympathetic nerves (Figure-3) (Sneddon & Burnstock, 1984; Stjarne & Astrand,
Depending on cellular conditions or stimulation frequency, NE, ATP and NPY are coreleased at different proportions in order to better control smooth muscle tone (Kasakov et al., 1988).

**Figure 3: Role of ATP and NE as Neuromodulators.** ATP and NE are coreleased from the same vesicle prejunctionally, and they can act as neuromodulators. At the postjunction, ATP acts on P2- purinoceptor receptor to caused blood vessel contraction, and NE binds to α1- adrenoceptor to prolong the contraction. These neurotransmitters can modulate its own release by binding to the α2- adrenoceptors and P1 purinoceptors located at the presjunctions (Burnstock, 1984).

These neurotransmitters can modulate the release of themselves and each other (Figure-3). For example, ATP can postjunctionally modulate and enhance the responses of the other co-transmitters. Prejunctionally, adenosine (ATP's metabolic breakdown product) was shown to modulate the release of noradrenaline from peripheral sympathetic nerves in a variety of tissues (Burnstock, 2009). NPY is usually co-stored with ATP and NE, and acts as a neuromodulator by prejunctionally reducing the release of NE and ATP, and/or by postjunctionally increasing the

Varying stimulation frequency can also modulate neurotransmitters released at the neuroeffector junction. Analyzing the kinetics of neuronally released co-transmitters from guinea pig vas deferens has shown that ATP and NE are released at different proportions depending on electrical frequency. ATP was released at low frequencies (8 HZ) of nerve stimulation. However, when stimulated at higher frequencies (16HZ or more), NE was released along with ATP after 10s of stimulation. It was noted that arterial smooth muscle contracted more at the frequencies in which NE was released. These results suggest that ATP and NE could be stored in different vesicles (Todorov et al., 1999).

Additional mechanisms have now been identified that control constriction and vasodilatation of arteries; for example, neuromodulators have the ability to alter the release and actions of other neurotransmitters. Known neuromodulators include circulating neurohormones, prostanoids, bradykinin, histamine, endothelin, in addition to neurotransmitters themselves (Milner et al., 1999). Neurotransmitters released at the neuroeffector junction can be modulated by non-neuromediators, such as the vasoactive peptide endothelin ET-1. Prejunctionally, endothelin-1 modulates the release of NE to decrease sympathetic neurotransmission. Numerous experiments have shown the modulation of sympathetic neurotransmission by ET-1, 6 such experiments were performed in the guinea pig pulmonary and femoral arteries (Wiklund & Cederqvist, 1989), rat and guinea pig vas deferens (Wiklund et al., 1990), dog coronary artery (Aarnio et al., 1993) and rat mesenteric artery (Aarnio et al., 1993). In the rat mesenteric bed, ET-1 negatively modulates the release of NPY instead of NE at the neuroeffector junction (Hoang et al., 2002). ET-1 regulates sympathetic neurotransmission postjucntionally by
enhancing vasoconstriction caused by both nerve stimulation and a variety of vasoactive agents (Henrion & Laher, 1993; Hoang et al., 2002). As ET-1 binds to the ETₐ receptors, it potentiates the postjunctional contractile effects of ATP (Mutaflova-Yambolieva & Radomirov, 1993; Hoang et al., 2002).

Hypertension has no single cause, and has been linked to various factors. Some of these factors are structural and functional changes in the vasculature. Medial smooth muscle hypertrophy and hyperplasia are all symptoms of hypertension. The three major neurotransmitter/neuromodulators NE, NPY, and ATP in the sympathetic nervous system have the ability to trigger a mitogenic response in human vascular smooth muscle cells (Thulin & Erlinge, 1995). In the spontaneously hypertensive rat (SHR) model, ATP was shown to regulate vascular smooth muscle cell proliferation (Harper et al., 1998). Other factors include the sympathetic nervous system, the rennin-angiotensin system and other endothelial irregularities (Milner et al., 1999). Animal models have become particularly useful in mapping the vascular changes that lead to the onset of hypertension. The SHR model has become very useful for understanding genetically role of hypertension. Using this model it has become possible to look at the involvement of the sympathetic nervous system in the origin and continuation of hypertension in SHR. An increase in the density of cerebral artery innervations by sympathetic nerves containing NE and NPY was shown to precede the onset of hypertension and related medial hypertrophy. After looking at sympathetic neurotransmission in the tail and mesenteric arteries of SHRs it has been suggested that ATP plays a greater role in co-transmission than NE. So much so, that ATP is the main component of the sympathetic response (Vidal et al., 1986).

Thus, enhanced sympathetic activity has been implicated in the pathogenesis of hypertension in humans.
PROJECT PURPOSE

Endothelin has been shown to modulate the release of the neurotransmitters NE and NPY at the neuroeffector junction. Previous studies on NGF differentiated PC12 cells suggest that ATP levels (a key modulator of vasoconstriction) were modulated by ET-1 (Gardner et al., 2005). It was shown that the ET_B receptor was able to inhibit the release of ATP by “a specific associated G-protein belonging to the Gi/o family, and attenuation of calcium levels” (Gardner et al., 2005). Notably, ATP release is the factor that causes contraction of vascular smooth muscle cells, and as a result it also regulates vascular resistance (Mutafova-Yambolieva & Radomirov, 1993). Therefore, Gardner’s hypothesis states that activation of the ET_B receptor by ET-1 recruits specific receptor associated G-proteins belonging to the Gi/o family, which also attenuates calcium levels. The enzymes and proteins involved in the signaling pathway that causes the release of ATP are not fully known. Therefore, the objective of this project is to identify some of the mediators utilized by the ET_B receptor and demonstrate their function in the regulation of neurotransmitter release. The knowledge gained from the ET_B signaling pathway is crucial for the development of a more specific endothelin-targeted therapy for PAH (Gardner, 2008).
METHODS

Cell Culture

Undifferentiated rat pheochromocytoma (PC12) cells were obtained from Professor Alice Gardner’s lab at the Massachusetts College of Pharmacy and Life Science. After being thawed, cells were transferred into T75 flask (polystyrene, Corning, New York) containing growth medium [Dulbecco’s Modified Eagle’s Medium (DMEM with 4.5 g/L glucose and 4.0 mM L-glutamine, Lonza) supplemented with 10% (v/v) horse serum (heat-inactivated at 55°C, 30 min), 5% (v/v) fetal bovine serum, and a penicillin streptomycin solution (100 U penicillin and 100 µg streptomycin/mL, Sigma)]. Cells were grown in a NAPCO 5400 series incubator at 37°C, 5.0% CO₂. After 24 hours, the cells were differentiated via the addition of mouse nerve growth factor (2.5 S NGF, BD Bioscience, 50 ng/mL). Medium was replaced every 2-3 days with growth medium containing NGF, as before. At 80% confluency, cells were subcultured and transferred into six well plates (polystyrene, Corning, New York).

Cell Stimulations

When cells reached confluence (70%), the growth media were replaced with DMEM media (4.5 g/L glucose & 4.0 mM L-glutamine, Lonza) supplemented with mouse NGF (BD Bioscience, 50 ng/mL). Cells were serum starved for 24 hours to achieve base line activity. The agents used to stimulated cells were BQ 3020 (10 mM; ET₄ agonist), U73122 (5 µM; PLC inhibitor), and KCl (50 mM). The control set was not treated with any of the agents. The first set of wells was basal and not stimulated. The second set was stimulated for 5 minutes with KCl. The third set was incubated for 5 minutes with BQ3020, and then stimulated for 5 minutes with
KCl. And finally the last set was initially pretreated for 20 minutes with U73122 then incubated for 5 minutes with BQ3020, and finally stimulated for 5 minutes with KCl. All cells were incubated in a NAPCO 5400 series incubator.

**Preparation of Cell Lysates**

Following stimulation, all media were aspirated, and the cells were washed with phosphate-buffered saline (ice-cold 1X PBS, without calcium & magnesium, Cellgrow). Lysis buffer containing 1X RIPA buffer (Pierce, Prod. # 89900) supplemented with 1x Halt Protease and phosphatase inhibitor cocktail (Pierce, 100x) was used to lyse cells. All cells were kept on ice, and incubated for 15 minutes to facilitate cell lysis. Using a cell scraper and pipetter the lysate was removed from each well and placed in a microcentrifuge tube. Inhibitor cocktail (40 µL) was used to wash each well. The wash from each well was added to the corresponding microcentrifuge tube containing cell lysate. This was incubated for 30 minutes. Cell lysate was centrifuged at 10,000xg for 10 minutes at 4°C. The supernatant was collected from each tube.

The total protein concentration in the supernatant was determined by the manufacturer’s microplate procedure obtained from the Pierce BCA™ Protein Assay Kit. Readings were obtained from a Synergy HT microplate reader. The BSA standard curve was made according to table 1, in the BCA™ Protein Assay Kit procedure pamphlet from Pierce thermo scientific. A modification to the table was made to extend the range of BSA standard concentration curve from 2,000 µg/mL – 25 µg/mL, to 2,000 µg/mL - 12.5 µg/mL.
**Electrophoresis**

Samples were added to Lane Marker Sample Reducing Buffer (Pierce, cat# 3900) at a ratio of 1 to 4 to make a 1X solution. The solution was boiled for 5 minutes. The total protein from each sample (20 µg), Trichromranger™ Prestain Protein (10 µL), Prosieve®Color Protein Marker (Lonza, Cat. No 50550), and Prosieve®Color Protein Marker (10 µL; Cambrex, Cat. No 50552) were loaded into PAGE® Gold Precast Gel (Lonza, 4-20% Tris-Glycine polyacrylamide Gel). Initially the gels were run in electrophoresis buffer (Pierce, 25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) at 125V for 30 minutes, then changed to 200V for the remainder of the run.

**Immunoblots**

Proteins were transferred onto PVDF membrane (BioTrace™ PVDF Polyvinylidene Floride Transfer membrane 0.45 µM, Pall Corporation Life Science) at 100 V for 1 hour or 30V overnight in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.0, Pierce) at 4°C. Memcode™ Reversible Protein Stain Kit (Pierce) was used to confirm the efficiency of protein transfer. Membrane was blocked with Starting Block™ T20 Blocking buffer (10 mL; Tris buffer saline containing 0.05% Tween-20, Pierce) for 30 minutes at 37°C, and then washed with 1X PBS (0.1% Tween™) for 15 minutes.

The antibodies were titrated under different concentrations to determine the optimal band contrast. The primary antibody (PLC-β1 rabbit polyclonal IgG, Santa Cruz, 200 µg/mL) was diluted 100 and 500 fold with blocking buffer (T20, pierce) up to 10 mL. The membrane was then incubated at room temperature for 1 hour with the primary antibody. The secondary antibody (Bovine anti–rabbit IgG-HRP, Santa Cruz, 200 µg/0.5 mL) was diluted 500 and 2000 fold with T20 blocking buffer (Tris buffer saline containing 0.05% Tween-20, Pierce) up to 10
mL. The membrane was then incubated at room temperature for 1 hour with the secondary antibody following a 15 minute 1X PBS wash (0.1% Tween™). Bands of interest were detected using chemiluminescence (Pierce® ECL Western Blotting Substrate, Pierce) and analyzed as a high-resolution scanned image of the film with the computer NIH program Scion Image.

**Immunoprecipitation of Phospholipase C**

Rat PC12 cells (passage number 22) were used for immunoprecipitation. Cells were treated with the same agents as before. Proteins were quantified using a BSA protein assay kit as before. The following steps were completed at 4°C.

Following cell lysis, the supernatant (containing 100 µg of protein) was pre-cleared before being used for immunoprecipitation. First, the volume of the lysate was brought up to 1 mL per vial of solution using 1x RIPA and Hal™ Protease & Phosphatase Inhibitor Cocktail 1X (Pierce). To the supernatant control IgG (0.25 µg; normal rabbit IgG, 200 µg/0.5mL, Santa Cruz) and suspended agarose conjugate (20 µL; Protein A/G PLUS- Agarose, Santa Cruz) were added to each vial. The vials were tumbled using a shaker (model 55 rocking shaker, Midwest Scientific) for 30 minutes at 4°C. The supernatant was then collected by centrifuging at 1,000xg for 30 seconds. The primary antibody (5 µL; PLC-β1 rabbit polyclonal IgG, Santa Cruz) was added to the supernatant and tumbled using the rocking shaker for two hours at 4°C. Afterwards, 20 µL of the same protein A/G-agarose was added to the solution and tumbled overnight at 4°C under the same conditions as the previous step. The pellet was collected by centrifuging at 1,000xg for 30 seconds. The pellet was washed and centrifuged (1,000xg for 30 seconds) three times using RIPA buffer (300 µL; Pierce) each wash. On the final wash, the resuspended pellet solution was centrifuged under the same conditions as before. The pellet was collected. The
beads were resuspended in electrophoresis sample buffer (2x, 40 µL; ProTrack™ Loading buffer Lonza; DTT 0.1 M Invitrogen, and 50 mM Tris-HCl 1% SDS). The samples were boiled for 3 minutes, and centrifuged at 1000xg for 30 seconds. The supernatant (40 µL) was collected from each tube, subdivided into two 20 µL aliquots, and stored at -80°C.

**Western-blot of Immunoprecipitated Phospholipase C**

The samples (20 µL) and marker (ProSieve® Color Protein Marker, Lonza, Cat. No. 50550) were loaded into 8-16% Precise™ Protein SDS-PAGE gels (Prod. No. 25203). The gel was run at 125 V in BupH™ Tris-HEPES-SDS running buffer (100 mM Tris, 100 mM HEPES and 3 mM SDS at pH 3±0.5, Pierce) at 4°C. The proteins were transferred onto PVDF membrane (BioTrace™ PVDF Polyvinylidene Fluoride Transfer membrane 0.45 µM, Pall Corporation Life Science) at 30V overnight in transfer buffer (25 mM Tris, 192 mM glycine, pH 8.0, Pierce) at 4°C.

The PVDF membrane was blocked with Starting Block™ T20 Blocking buffer (10 mL; Tris buffer saline containing 0.05%Tween-20, Pierce) for 30 minutes at room temperature on a rocking machine (Jahre Garentie, Heidolph Rotamax 120). Anti-Phosphotyrosine (4G10®) HRP Conjugated monoclonal antibody (Millipore, Cat# 16105) was diluted 1:1000 using the Starting Block™ T20 Blocking buffer. The PVDF membrane was incubated in the antibody at 4°C overnight on the rocking machine. The PVDF membrane was washed twice with water, then T20 blocking buffer for 5 minutes and finally with water 5 times. The bands were detected according to the previous method.
Quantifying ATP Release

Cells were grown and differentiated as before. On the day of the experiment the media was replaced with Krebs’ buffer (1.5 mL per well) composed of NaCl (119 mM), KCl (2.5 mM), MgSO₄ (1.3 mM), CaCl₂ (2.5 mM), NaH₂PO₄ (1.0 mM), NaHCO₃ (26.2 mM), and HEPES (10 mM) at pH 7.4. The plates were allowed to equilibrate in a shaking water bath at 37°C for 15 minutes. Cells were stimulated with BQ 3020 (10 mM; ETₐ agonist), ET-1 (10⁻¹⁰ M), U73122 (5 µM; PLC inhibitor), and KCl (50 mM) in the same manner as before. ET-1 (10⁻¹⁰ M) was stimulated in a similar way as BQ 3020 (10 mM; ETₐ agonist). After stimulation, plates were placed on ice. The buffer and cells were removed from plates as before, and centrifuged at 1000 rpm for 2 min. The supernatant was used for quantifying ATP (Gardner et al., 2005).

Analysis of Purines

The following method used to analyze purines was described by (Levitt et al., 1984). Chloroacetaldehyde was synthesized according to the methods described by (JA et al., 1972). This compound was used to form fluorescent 1, N6-ethenopurine analogs (E-purine). E-purines can be “simultaneously separated from the sample by reverse-phase HPLC and quantified by fluorescent detection “ (Gardner et al., 2005). Chloroacetaldehyde (50 uL) was incubated with the supernatant (from quantifying ATP release) at 80°C for 40 minutes in a dry bath. Placing the samples on ice stopped the reaction. Afterwards, the samples were analyzed by HPLC-fluorometric detection. The different purine analogs formed were separated by a reverse-phase C-18 column. Two different buffers were used to create a dual buffer gradient system which could be used to separate and elute the purines from the column. Buffer A was composed of phosphate buffer (0.1 M, pH 6.0) and buffer B was composed of 75% 0.1M phosphate buffer and
25% methanol at pH 6.0. The dual buffer gradient system was created by gradually increasing the concentration of buffer B while decreasing the concentration of buffer A. A Varian 9070 Fluorescence Detector was set at an excitation wavelength of 300nm, and an emission wavelength of 420 nm, in order to detect the fluorescent purine derivatives. Carrying out a comparison against purine standard retention times identified the purine peaks. The purine content of the sample was quantified using the Varian Star Workstation Software to integrate the purine peaks. “ATP analysis was performed on perfusates and normalized against protein concentrations” (Gardner et al., 2005).
RESULTS

NGF-differentiated PC12 cells were used in this study of the signaling mechanism utilized by the endothelin-1 (ET-1) modulation of sympathetic neurotransmission. Since ET-1 is an important vasomodulating hormone, understanding its mechanism of action is important for designing drugs to treat hypertension diseases. Analyzing the signaling pathway of the ET$_B$ receptor has given insight into its role in pulmonary arterial hypertension (PAH). It was previously shown that ET-1 binding to the ET$_A$ receptor resulted in phosphorylation of PLC, which caused the levels of 1,4,5-triphosphate (IP$_3$) and diacylglycerol to increase (Senogles, 1994). This increased level of IP$_3$ in turn caused the intracellular calcium concentration to increase. In smooth muscle, studying the ET-1 pathway revealed that phosphorylation of PLC caused intracellular calcium to increase transiently (Takuwa, 1990). In this project, treatment of PC12 cells with PLC inhibitors, or ET-1, or other ET$_B$ agonists were used to test whether PLC kinase plays a role in ET$_B$-mediated inhibition of ATP release.

Phospholipase C Modulates Endothelin-Mediated ATP Release Via the ET$_B$ Receptor

In order to determine whether phospholipase C (PLC) modulates the release of ATP from PC12 cells, the effects of a PLC antagonist on the K$^+$ evoked release of ATP from NGF-differentiated PC12 cells were studied to show whether this agent could reverse the ET-1 induced inhibition on the K$^+$ evoked release of ATP (Figure-4). Stimulation of NGF differentiated PC12 cells with KCl (50 mM) (first histobar) caused ATP to release over basal levels. Adding ET-1 (second histobar) reduced ATP levels. The addition of the PLC inhibitor (U73122, shown as U7 in the figure) (third histobar) reversed the ET-1-induced inhibition on the
K⁺ evoked release of ATP; however, the changes in ATP levels were not significant. Thus, this data shows that in PC12 cells, ET-1 may act to lower KCl-induced ATP release, and this release appears to require PLC.

Figure 4: The Effect of ET<sub>AB</sub> Agonist ET-1 and Phospholipase C Inhibition on Stimulated KCl Induced ATP Release. Undifferentiated rat pheochromocytoma (PC12) cells were grown to 70-80% confluency and were differentiated by mouse NGF (50 ng/mL). Cells were serum starved for 24 hours and were stimulated with U73122 (5µM), ET-1 (10-10 M), or KCl (50 mM) accordingly. Treatment with the ET<sub>AB</sub> agonist ET-1 reduced ATP levels compared to KCL-stimulated ATP release. While in the presence of U73122, ET-1 modulated KCL-stimulation increased ATP release. Both of these changes were non-significant. Values are shown relative to untreated samples. Histobars denote the mean of 3 experiments. Error bars denote standard error.

Because ET-1 binds with equal affinity to the ET<sub>A</sub> and the ET<sub>B</sub> receptors, the effect of the ET<sub>B</sub> receptor by itself on PLC activation could not be clearly delineated. Therefore, the ET<sub>B</sub> agonist (BQ3020) was tested in conjunction with the U7 PLC inhibitor to further elucidate the role of PLC in ATP release mediated by the ET<sub>B</sub> receptor (Figure-5). NGF-differentiated PC 12 cells were stimulated with KCl (50 mM) to evoke ATP release above basal level (first histobar). The ATP levels decreased 30.7% under the presence of the ET<sub>B</sub> receptor agonist BQ3020 (second histobar). The PLC inhibitor U73122 reversed this inhibition by evoking a 73.5%
increased in ATP release (third histobar). Thus, receptor ET$_B$ indeed appears to participate in the lowering of KCL-induced ATP release.

Figure 5: The Effect of ET$_B$ Agonist and Phospholipase C Inhibition on KCl Induced ATP Release. Undifferentiated rat pheochromocytoma (PC12) cells were grown to 70-80% confluency and were differentiated by mouse NGF (50 ng/mL). Cells were serum starved for 24 hours and were stimulated with U73122 (5 µM), BQ 3020 (10 mM), or KCl (50 mM) accordingly. After stimulation with KCl, ATP levels decreased (30.7%, p<0.05) under the presence of the ET$_B$ receptor agonist, BQ-3020, compared to KCl stimulation only. The inhibitory effects of BQ-3020 on ATP release was reversed after pretreating with the non-specific PLC inhibitor U73122 (73.5% increase, p<0.05). Each bar represents the mean ±S.E.M of 12 to 15 from six individual experiments. *, P< 0.05 KCl vs. KCl+ BQ3020 and KCl+ BQ3020 vs. KCl +BQ3020 +U73122. Error bars denote standard error.

In order to determine whether PLC plays a significant role in modulating basal ATP release, BQ 3020 or U73122 were incubated with control cells (Figure-6). The BQ3020 (shown as BQ in the figure) ET$_B$ agonist (second histobar) had no significant impact on basal ATP levels. However, the addition of the U73122 PLC-inhibitor (third histobar) resulted in a significant increase in the basal level of ATP release, suggesting that PLC plays a role in modulating basal neurotransmitter release.
Figure 6: The Effect of ET<sub>B</sub> Agonist and Phospholipase C Inhibitor on Basal ATP Release. Undifferentiated rat pheochromocytoma (PC12) cells were grown to 70-80% confluency and were differentiated by mouse NGF (50 ng/mL). Cells were serum starved for 24 hours and were incubated in either BQ 3020 (10 mM; 5 min) or U73122 (5 µM; 20 min). The BQ ET<sub>B</sub> agonist (second histobar) did not have a significant impact on basal ATP level, but inhibiting PLC with U7 significantly increased ATP release (third histobar). Therefore, PLC significantly modulates basal ATP release. Each bar represents the mean ±S.E.M of 12 to 15 from six individual experiments. *, P< 0.05 Basal + BQ3020 vs. Basal + U73122. Error bars denote standard error.

Quantifying PLC Phosphorylation Via Western Blotting

After determining that PLC plays a role in ATP release, it became important to figure out which PLC family and isotype takes part in the ET<sub>B</sub> signal transduction pathway. U73122 has been shown to only inhibit PLC-β and PLC-γ (Heemskerk et al., 1997). PLC-β was chosen over other PLC family members because it is involved in the IP3 pathway. Cellular levels of PLC-β were monitored by immunoblot (Figure-7). For each lane there was only one PLC-β1 band instead of a doublet, as one would expect from a phosphorylated enzyme in a western blot. The primary antibody (in the immunoblots section under methods) is capable of detecting both phosphorylated and unphosphorylated PLC-β1. The phosphorylated PLC-β1 should appear as a slightly heavier band above the unphosphorylated band. Because this assay was not sensitive enough, a doublet was not seen.
Figure 7: The Effect of ET<sub>B</sub> Agonist (BQ3020) on PLC Phosphorylation.
Undifferentiated rat pheochromocytoma (PC12) cells were grown to 70-80% confluency and were differentiated by mouse NGF (50 ng/mL). Cells were serum starved for 24 hours and were stimulated with BQ 3020 (10 mM), and KCl (50 mM) as listed. Primary antibody detection (5 µL; PLC-β1 rabbit polyclonal IgG, Santa Cruz diluted 1:500 in Tris buffer saline containing 0.05%Tween-20, Pierce) and secondary antibody (Bovine anti –rabbit IgG-HRP, Santa Cruz, 200µg/0.5 mL; diluted 1:2000 in Tris buffer saline containing 0.05%Tween-20, Pierce).
DISCUSSION

In the sympathetic nervous system, endothelin-1 (ET-1) was previously shown to act as a neuromodulator in experiments done on the guinea pig pulmonary and femoral arteries (Wiklund & Cederqvist, 1989), rat and guinea pig vas deferens (Wiklund et al., 1990), dog coronary artery (Aarnio et al., 1993) and rat mesenteric artery (Aarnio et al., 1993). Specifically, when studying the effects of ET-1 on the endothelial ET\textsubscript{A} receptor, ET-1 was shown to potentiate the postjunctional contractile effects of ATP (Mutafova-Yambolieva & Radomirov, 1993; Hoang et al., 2002). Notably, ATP release is the factor that causes contraction of vascular smooth muscle cells, and as a result regulates vascular resistance (Mutafova-Yambolieva & Radomirov, 1993). So understanding this pathway is important for the future design of drugs to treat hypertension disorders.

Previous data showed that the ET\textsubscript{B} receptor was able to inhibit the release of ATP by “a specific associated G-protein belonging to the Gi/o family, and attenuation of calcium levels” (Gardner et al., 2005). Calcium therefore plays an essential role in neurotransmitter release. As previously discussed, calcium can enter the neuron via the opening of the voltage dependent calcium channels. Neurotransmitter release can also be altered by modulating calcium release, for example by inhibiting voltage-dependent calcium channels by activation of the PLC/Ca(2+)-dependent PKC signal transduction pathway (Salgado et al., 2007).

The Activation of the PLC/Ca(2+)-dependent PKC signal transduction pathway leads to PLC phosphorylation which in turn hydrolyzes PIP\textsubscript{2} to produce IP\textsubscript{3} and diacylglycerol (Hou et al., 2003). Furthermore, studying the endothelin pathway in smooth muscle revealed that phosphorylation of PLC caused intracellular calcium to increase transiently (Takuwa, 1990).
In our project, PLC inhibitors were used to test whether PLC plays a role in ET$_B$ mediated inhibition of ATP release. Cells were stimulated with the non-specific PLC inhibitor (U73122), ET$_B$ agonist (BQ3020), and/or KCl. When the ET$_B$ agonist BQ3020 bound to the ET$_B$ receptor, a decrease in ATP levels was observed. However, once the non-specific PLC inhibitor U73211 inhibited PLC, an increase in ATP levels was seen. KCl stimulation of ATP in the presence or absence of U73122 did not change ATP levels. This suggested that PLC plays a role in the ET$_B$ signal transduction pathway because it did not alter KCl-induced ATP levels. Moreover, KCl caused ATP levels to increase by acting on voltage gated calcium channels.

However, under basal conditions, ATP levels of cells that were challenged with ET$_B$ agonists only slightly decreased, whereas those that were treated with U73122 caused ATP levels to increase significantly. Therefore, PLC plays a role in modulating basal and ET$_B$-modulated ATP release.

U73122 has been previously used extensively as a PLC inhibitor in the study of cellular signal transduction (Wilsher, et al., 2007). However, this aminosteroid is not entirely specific to any PLC family but has been shown to inhibit both PLC-β and PLC-γ isoforms, but not other PLC families (delta, epsilon, zeta and eta) (Heemskerk et al., 1997). Furthermore, U73122 has been shown to have a higher affinity towards human PLC-β2 compared to the other PLC-β isoforms (PLC-β1, PLC-β3, and PLC-β4), although this specificity has not been confirmed with the rat PLC-β analyzed in our project (Hou et al., 2003).

The first PLC immunoblot did not show a doublet around 150 kD (Figure-7), as one would expect when detecting both phosphorylated and unphosphorylated PLC. The antibody used in our immunoblot is capable of detecting both forms. Since only a small portion of PLC gets phosphorylated during activation, we conclude that the immunoblot method was not
sensitive enough to detect the phosphorylated form. PLC phosphorylation occurs within the first few signaling cascading events, is transient, and is not heavily amplified, so the phosphorylated form exists at very low concentrations, making it difficult to detect by this method (Hou et al., 2003).

In order to enhance the sensitivity, an immunoprecipitation using PLC-β1 antibody followed by an immunoblot with phosphotyrosine antibody was performed. In this method, only phosphorylated PLC is detected (by the phosphotyrosine antibody), thus only one band around 150 kD would be expected in the blot. However, because of ongoing troubleshooting, this result is still pending. If the phosphorylation levels were to correspond to ATP levels, then one could conclude that PLC activation is involved in ET_B signal transduction.

If a decrease in ATP levels, upon ET_B stimulation, is not found to correspond to a decrease in PLC phosphorylation, then the following conclusions could be made. Firstly, the blot could again not be sensitive enough, and further study would be needed to develop a more sensitive assay (radio-nucleotide assay using ^32P would be employed). Secondly it could be that PLC-β1 does not take part in the rat NGF differentiated PC12 ET_B pathway. Finally it could be that PLC-β1 is phosphorylated by serine (Ryu, et al., 1990) as shown in other cellular PLC signal transduction. Eukaryotic kinases in addition to phosphorylating tyrosine residues can also phosphorylate serine/threonine motifs, but very rarely histidine and aspartate amino acid residues (IonSource, 2009). As this project focused solely on tyrosine phosphorylation of PLC-β1 an assay looking for serine/threonine phosphorylation would need to be undertaken. Therefore, the short-term goal of this project is to improve quantification of PLC-β1 phosphorylation.

Future directions would be to develop a PLC-knockdown protocol to determine whether a relationship exists between PLC-β1 phosphorylation and ATP release to support this assertion.
BIBLIOGRAPHY


Piuhola, J. (2002). Regulation of cardiac responses to increased load: Role of endothelin-1, angiotensin II and collagen XV. University of Oulu, Department of Pharmacology and Toxicology, University of Oulu Biocenter Oulu. Oulu: Oulu University Library.


