Identification of A New Pharmacological Modulator for P2Y2 Receptor Activity

by

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Abstract

P2Y2 is a G-protein-coupled membrane receptor activated by ATP or UTP nucleotides. They play major roles in cellular physiology in different aspects including inflammatory responses and apoptosis. Therefore, P2Y2 receptor is considered to be a potential therapeutic target for regression of vascular inflammation. Various UTP modifications have been developed in order to increase their selectivity and stability. Here, we observed that aminoallyl-UTP is a cell-specific biased ligand for P2Y2 receptor. Interestingly, aminoallyl-UTP mediated Ca^{2+} signaling only in hP2Y2-transfected 1321N1 astrocytoma cells in a dose-dependent manner, but not in HCAEC and HeLa cells which express a high level of endogenous P2Y2 receptor. Aminoallyl-UTP-activated P2Y2 receptors were seen to increase phosphorylation of Akt, but with no effect on MAPK pathways, whereas UTP inhibits Akt phosphorylation and activates MAPK pathways in HCAEC. Our study provides new evidence that the P2Y2 receptor can be pharmacologically manipulated to target desired pathways in a cell-specific manner.

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CRP	C-reactive protein
ERK	Extracellular signal-regulated kinase
ET-1	Endothelin
FOXK1	Forkhead box K1
GPCR	G-protein coupled receptor
GRK	G-receptor kinase
HBP	Heparin-binding protein
ICAM-1	Intracellular cell adhesion molecule-1
IL	Interleukin
JNK	c-Jun NH2-terminal kinase
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemotactic protein-1
mTORC1	Mammalian target of rapamycin complex-1
NF-κB	Nuclear factor-kappa B
PDK-1	phosphoinositide-dependent kinase-1
PI3K	Phosphatidylinositol triphosphate kinase
PKA	Protein kinase A
PKC	Protein kinase C
PKD	Protein kinase D

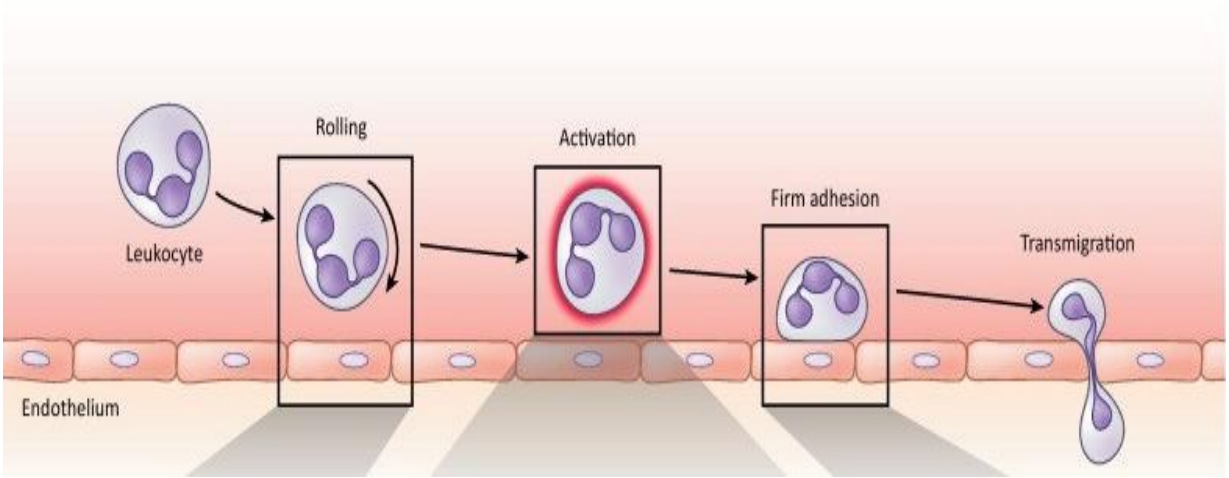
PLC	Phospholipase C
PMNs	Polymorphonuclear neutrophils
PSGL-1	P-selectin glycoprotein ligand-1
TNF-α	Tumor necrosis factor-alpha
VCAM-1	Vascular cell adhesion molecule-1

Chapter 1. Literature Review

1.1. Vascular Inflammation

According to the CDC, there are about 630,000 people who die every year in the United States because of cardiovascular disease (Benjamin et al, 2017). Atherosclerosis, a disease caused by lipids accumulation in large arteries, contributes to most cardiovascular diseases and stroke. The link between hypercholesterolemia and atherosclerosis was extensively studied before 1970's. However, with better understanding of vascular biology, vascular inflammation plays a major role in initiation and progression of atherosclerosis (Lusis, 2000). Although vascular inflammation is a natural protective response to endothelium injury, uncontrolled inflammatory responses can disturb normal functions of endothelium which are to maintain fluidity of blood and to control the entry of leukocytes into underlying tissues. Vascular inflammation, also known as vasculitides, can be divided into either infectious or non-infectious vasculitides. It is characterized by imbalance in endothelial homoeostasis and immune cells infiltration. Therefore, vascular inflammation results in edema at the site of lesion. Generally, activated endothelial cells express several receptors and release signaling molecules and chemoattractants such as cytokines in order to recruit leukocytes at the site of inflammation in a process known as leukocyte trafficking and adhesion. After activation and adhesion, leukocytes migrate into the underlying tissue and release signaling chemokines (**Figure 1.1**).

Figure 1.1



Leukocyte response in inflammation (adapted from Hajishengallis & Chavaki, Trend Immunol **34**, 2012):

Leukocytes are recruited to the site of inflammation followed by rolling, adhesion, and transmigration.

1.1.1. Leukocyte Rolling and Adhesion

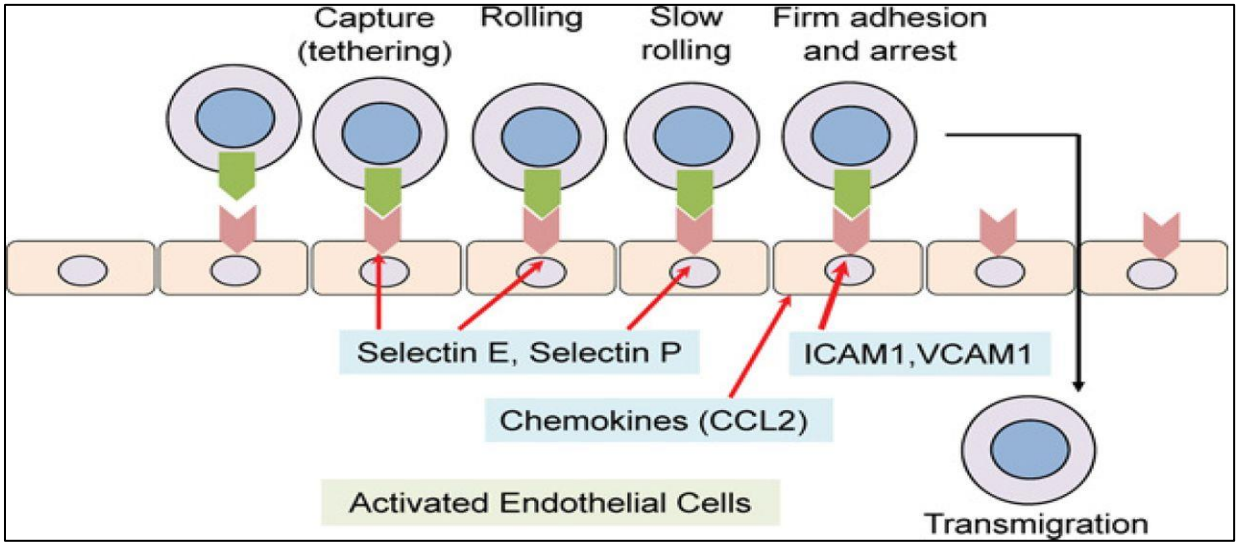
Leukocytes' ability to migrate and interact with activated endothelial cells is essential in acute vascular inflammation. The adhesion cascade is highly regulated and controlled to ensure an effective response. There are various overlapping processes that involve different leukocytes, cell adhesion molecules, chemokines, and cytokines. Platelets are also involved in vascular inflammation. The first leukocytes that migrate to the site of inflammation are polymorphonuclear (PMN) neutrophils (Witko-Sarsat et al, 2000). Neutrophils recruitment is mainly mediated by

released interleukin-8, IL-8, (de Oliveira et al, 2016), leukotriene B4, and complement protein 5a (Wagner & Roth, 2000). The activated PMNs influence the recruitment of a second influx of monocytes by releasing several granules at the inflammation site such as cathelicidin LL-37 and heparin binding protein (HBP). Cathelicidin LL-37 is 37-amino-acid antimicrobial peptides. Beside its antimicrobial activity in disturbing bacterial membranes (Braff & Gallo, 2006), LL-37 along with HBP attract specifically inflammatory bone marrow-derived monocytes to the inflammation site by activating formyl-peptide receptors expressed on activated endothelial cells (Soehnlein et al, 2008). There are two subsets of monocytes classified based on expressed chemokine receptors: inflammatory ($Ly6C^{high}$, $CCR2^{+}$, $CXCR1^{+}$) and resident ($Ly6C^{low}$, $CCR2^{-}$, $CXCR1^{++}$) monocytes (Geissmann et al, 2003). HBP induces the expression of monocyte chemotactic protein-1 (MCP-1) by activated endothelium. HBP-mediated MCP-1 expression is achieved by HBP sequential activating of Akt and p38 pathways via phosphorylation. HBP also activates MCP-1 transcription factor, NF-κB (Chang et al, 2017). Activation of MCP-1 explains the specific attraction of inflammatory monocytes that leave the bone marrow in a $CCR2^{+}$-dependent manner. CCR2, CC-motif chemokine receptor-2, binds to MCP-1 (Soehnlein et al, 2008). Monocytes are recruited at two distinct time phases after neutrophil cellular cross-talk: early after 3 hours and late at 24 hours, but MCP-1 concentration remains the same in neutropenic and neutrophilic mice (Janardhan et al, 2006). The investigators concluded that monocyte recruitment is dependent on neutrophils but not MCP-1. However, although not fully understood, activation of MCP-1expression is multifactorial involving various mediators. MCP-1 gene expression can be induced by tumor necrosis factor (TNF)-α (Murao et al, 2000). Another MCP-1 regulator is mammalian target of rapamycin complex 1 (mTORC1), which induces dephosphorylation of transcription factor forkhead box K1 (FOXK1) to activate transcription of MCP-1 gene in an NF-

κB independent manner (Nakastumi et al, 2017). Additionally, C-reactive protein (CRP) which is a hepatic protein produced in response to inflammation can promote release of endothelin, ET-1, which upregulates the expression of MCP-1 (Verma et al, 2002). Moreover, gC1qR/p33 protein also plays an important role in MCP-1 production as it affects the production at translational/post-translational levels (Anders et al, 2018). In essence, MCP-1 production is highly regulated and many factors are involved to ensure efficient response.

Monocyte rolling adhesion is critical to inflammation initiation (**Figure 1.2**). Rolling adhesion is mediated by C-type lectins, known as P- and E-selectins, that bind carbohydrate ligands. P-selectins are expressed on endothelial cells and bind PSGL-1, P-selectin glycoprotein ligand-1, which is expressed on monocytes during rolling over epithelium. PSGL-1 and P-selectin interaction is essential in monocyte capture and slow rolling. The interaction is supported by E-selectins which are expressed by endothelial cells during inflammation and overlap with P-selectins (Sperandio et al, 2003). Once the interaction is established, monocytes use vascular cell adhesion molecule (VCAM-1), which is only expressed by activated endothelial cells, for slow rolling and tight adhesion. VCAM-1 expression is activated by NF-κB. Lin et al (2015) demonstrated that TNF-α activates tumor necrosis factor receptor-1 (TNFR-1) which then mediates c-Src signaling pathway. Thus, c-Src activates PI3K/Akt and NF-κB pathways. NF-κB then upregulates the expression of VCAM-1 gene. Another chemokine similar to VCAM-1 is intracellular cell adhesion molecule (ICAM-1) which is expressed by endothelial cells during inflammation and induced by NF-κB. However, unlike VCAM-1 which is expressed at the site of lesion, ICAM-1 is broadly expressed even in unaffected regions and involved in late stage of adhesion (Cybulsky t al, 2001). Recruited monocytes then differentiate into macrophages. Based on what cytokines they produce, macrophages are classified into two groups: M1 (pro-

Figure 1.2



Leukocyte rolling and adhesion (Wada & Makino, Clin Sci **124,** 2013):

P/E selectins binding to PSGL-1 captures rolling leukocytes. Tight adhesion is mediated by VCAM-1 and ICAM-1.

inflammatory) and M2 (anti-inflammatory). Differentiation into M1 pro-inflammatory macrophages is stimulated by TNF-α and/or bacterial lipopolysaccharides. On the other hand, M2 macrophages are produced in response to IL-4 and IL-13, which both act as feedback effectors. Granulocyte/macrophage colony stimulating factor (GM-CSF), beside its hematopoietic activities, is one of major pro-inflammatory mediators. It mediates monocyte differentiation into M1 pro-inflammatory macrophages that produce several cytokines such as IL-6, IL-12, IL-23, IL-1β, and TNF (Fleetwood et al, 2007). A recent study by Shirakura et al (2018) demonstrates the role of Roundabout4 (Robo4) in GM-CSF production. Robo4 is a receptor expressed on epithelial cells

during inflammation and activated by slit proteins (Huminiecki et al, 2002). Robo4 promotes production of GM-CSF by activated endothelial cells and therefore upregulates production of IL-6 and IL-1β. Monocyte-derived macrophages undergo self-renewal independently from monocytes and therefore become dominant at lesion site (Schulz et al, 2012).

Recruitment of lymphocytes also occurs at the inflammatory region. T lymphocytes rolling and adhesion are mediated by L-selectins expressed on lymphocytes (Galkina et al, 2006). Generally, L-selectins are expressed by all types of leukocytes and are involved in leukocyte rolling, slow rolling and capture, activation, and transmigration into underlying tissues during inflammation (Grailer et al, 2009). As being expressed on leukocyte surfaces, L-selectins also mediate secondary tathering via leukocyte-leukocyte interaction (Erikksson et al, 2001). B lymphocytes can also be found at the site of inflammation (Zhou et al, 1999).

1.1.2. Platelets

Platelets are involved in leukocyte recruitment in vascular inflammation. They secrete inflammatory mediators that recruit more leukocytes to the site of inflammation (von Hundelshaun & Weber, 2007). Platelets express P-selectins for lesion development and the interaction with PSGL-1 on monocyte mediate firm adhesion by activating macrophage-1 (Mac-1) antigen and very-late antigen-4 (VLA-4) integrins (Burger & Wagner, 2003). Therefore, aggregated platelets form a physical bridge between endothelium and leukocytes and produce mediators to initiate leukocytes accumulation.

1.1.3. Signaling Molecules (Cytokines)

TNF-α plays important roles in development of vascular inflammation. As mentioned above, TNF-α induces MCP-1 gene expression. It mediates PI3K/Akt signaling pathways via activating TNFR-1. Thus, TNF-α stimulates specific differentiation of monocytes into M1 macrophages. In

addition, TNF-α-deficient mice showed significant decrease in VCAM-1 and ICAM-1 expression (Otha et al, 2005).

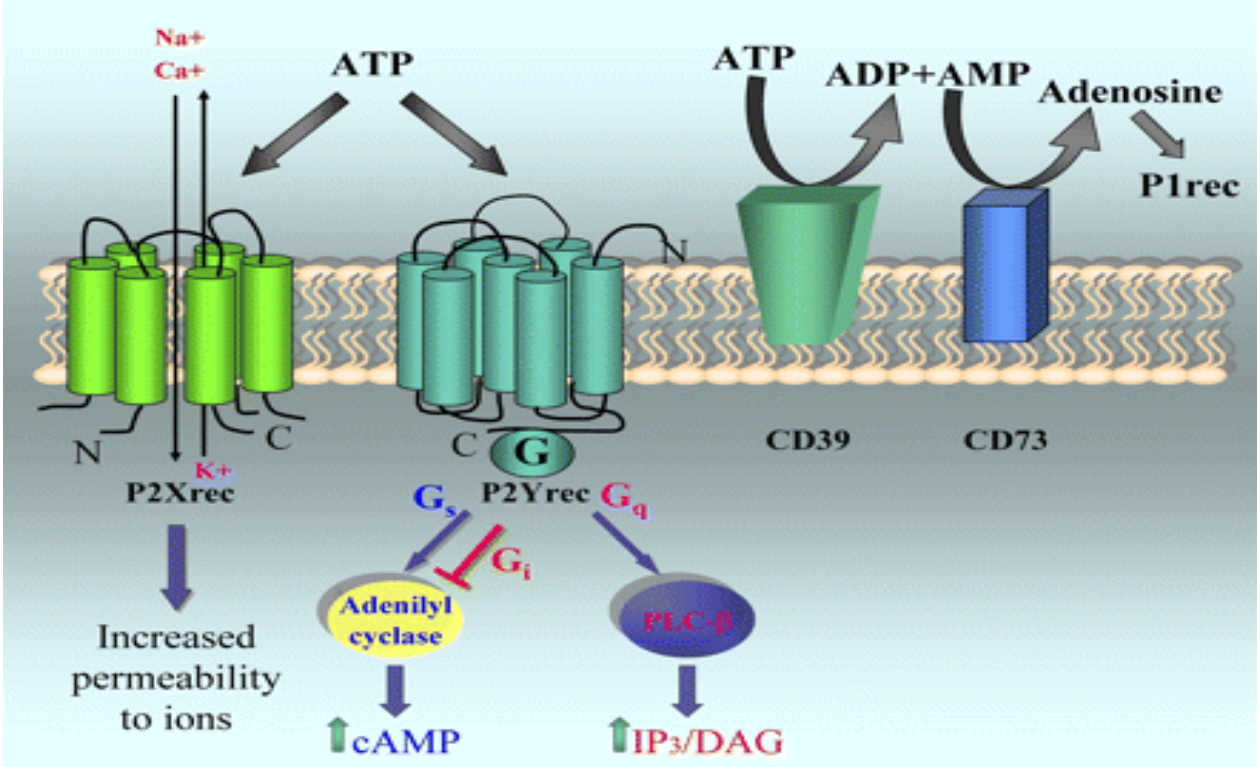
NLRP3 Inflammasome**,** Nucleotide-binding oligomerization domain (NOD)-like receptor, pyrin-containing domain 3 (NLRP3) complex, contains 3 proteins: NLRP3 receptor, apoptosis-associated speck-like protein containing caspase and activation recruitment domain (PYCARD), and cysteine protease caspase-1 (Martinon et al, 2009). Activation of NLRP3 is mediated by either pathogen-associated molecular pattern (PAMP) and/or (DAMP) damage-associated molecular patterns such as adenosine triphosphate (ATP) (Pope & Tschopp, 2007). Activated NLRP3 induces release of various cytokines such as IL-1β and TNF-α (Stewart et al, 2010).

IL-1β, interleukin-1β, is produced by macrophages and activated via caspase-1-mediated cleavage (Martinon et al, 2002). It was thought that production of IL-1β was mediated by TNF-α (Knudsen, 1986), but with further understanding, it has been shown that expression of IL-1β is upregulated by NLRP3 via TNF-α (Stewart et al, 2010). Active IL-1β binds its receptor (IL-1R1 and IL-1RAP) and induces expression of NF- κB and mitogen-activated protein kinase, MAPK (Sims & Smith, 2010).

IL-8, also known as CXCL8, is mainly produced by monocytes, macrophages, lymphocytes, and endothelial cells. IL-8 binds their specific receptors: CXCR1 and CXCR2 expressed on leukocytes (Bickel, 1993). It stimulates migration of neutrophils, monocytes, and macrophages to the inflammation site (de Oliveira et al, 2016). It also favors monocyte differentiation into M1 macrophages (Meniailo et al, 2018). IL-8 production can also be mediated by extracellular ATP-induced P2 receptors in monocytes to control neutrophil migration to the site of inflammation (Kukulski et al, 2009).

Extracellular Nucleotides, besides being DNA and RNA backbones, nucleotides are released extracellularly as signaling molecules. Extracellular nucleotides are released mainly in form of ATP, universal energy carrier, from cells under stress or activated during inflammation responses. Inflammatory cells release extracellular ATP through connexin hemichannels (Faigle et al, 2008). In apoptosis, stressed cells release ATP via pannexin hemichannels (Ravichandran, 2011). Due to abundance of nucleotidases on the cell membrane, ATP hydrolysis occurs in two-step enzymatic reactions catalyzed by ectonucleotidases. In the first step, ATP and ADP are cleaved into AMP by CD39, ectonucleoside triphosphate diphosphohydrolase family (E-NTPDase) (Robson et al, 2006). AMP is then broken into adenosine and a phosphate group via ecto-5'-ectonucleotidase (CD73). Deletion of CD39 causes attenuation in adenosine levels and increased ATP and ADP levels. However, CD39 knock out mice are viable but susceptible to inflammation (Eltzchig et al, 2009). On the other hand, knocking out *cd73* gene results in attenuation in adenosine but unchanged ATP and ADP levels; and mice are susceptible to inflammation. (Thompson et al,2004). These nucleotides mediate activation of purinergic receptors: P1 and P2 receptors (**Figure 1.3**). P1 receptors are G-protein coupled and sensitive to adenosine. On the other hand, P2 receptors are G-protein coupled sensitive to ATP, ADP, UTP, and UDP (Alexander et al, 2015). They are subdivided into two groups: ionotropic P2X and metabotropic P2Y.

Figure 1.3



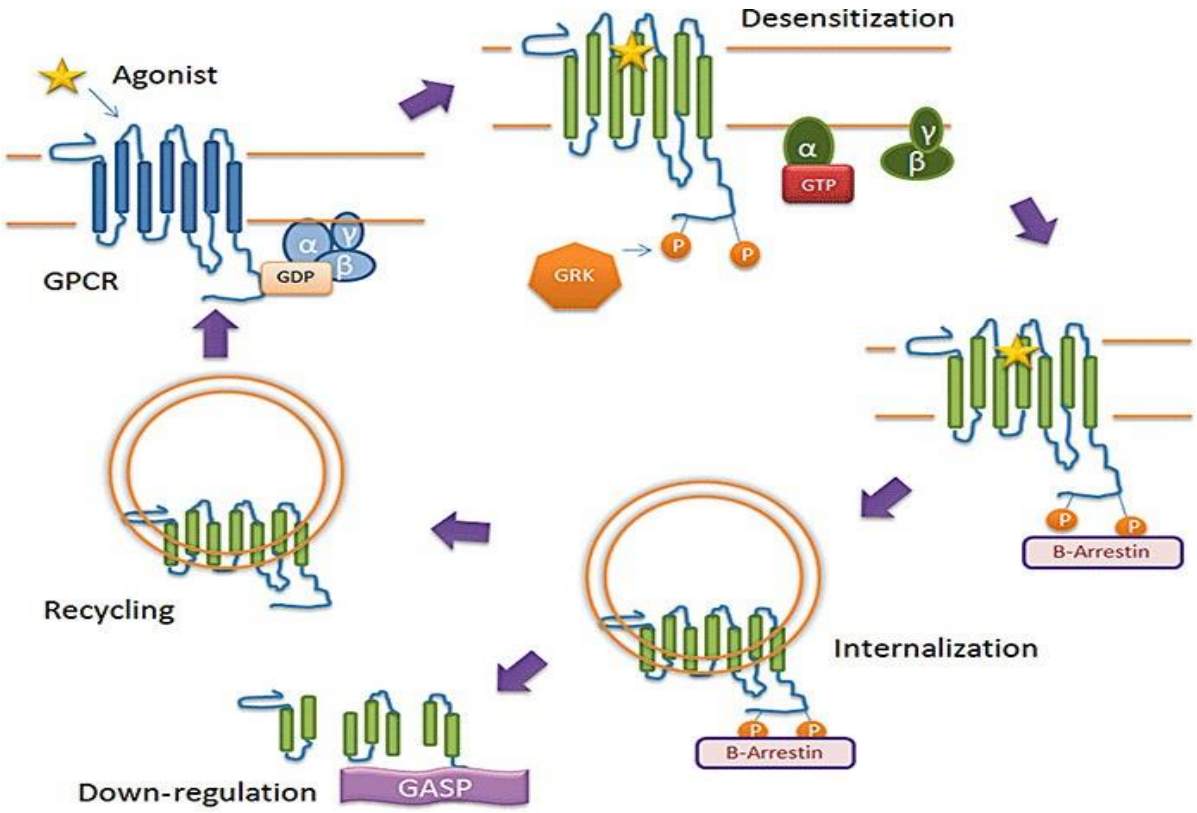
ATP processing at the cell membrane (Vitiello et al, Blood **120**, 2012):

Activated P2X receptors flex and open to become permeable for Na^+ and Ca^{2+} influx coupled with K^+ efflux. G_q-coupled P2Y receptors activate PLC to breakdown PIP2 into IP3 and diacylglycerol. ATP hydrolysis to ADP and then AMP is mediated by CD39. AMP is cleaved into adenosine (P1 receptor stimulator) by CD73.

1.2. G Protein-coupled Receptors (GPCR)

GPCRs are the most abundant receptors expressed on mammalian cells. The G protein is composed of α, β, and γ subunits. These receptors receive extracellular signals and transduce the signal intracellularly through mediating second messengers' activation such as increasing calcium release from Ca^{2+} stores or increasing cAMP production. The inactive form has GDP bound to the α subunit that has a GTPase activity. Upon activation, a conformational change results in release of GDP and binding of GTP. The complex of GTP-G_α and $G_{\beta\gamma}$ are released and mediate downstream signaling (Cabera-Vera et al, 2003). Based on differences in the Gα subunit and effectors the protein interacts with, G proteins are classified into four subtypes: G_q, G_s, $G_{i/o}$, and $G_{12/13}$. Receptor coupling to a specific G protein subtype defines the effectors and pathways the G protein activates or deactivates. G_q-coupled receptors activate phosphorylation of phospholipase C. G_s-coupled receptors activate adenylyl cyclase which in turn converts ATP to cAMP, while G_i inhibits the conversion (Erb and Weisman, 2012). Signaling to or through GPRCs has to be very regulated and controlled because GPRCs are the most abundant receptors on mammalian cells. The modulation of receptor activity starts with receptor desensitization, a reduction in the receptor response to activators or agonists regardless continuous stimulation. The receptor is phosphorylated by protein kinases (PKA and PKC) or by G-receptor kinase (GRK). The phosphorylation enhances the binding of β-arrestin (Gurevick et al, 2006). There are two sites where β-arrestin binds: at the terminal C-domain tail or at the receptor transmembrane core. Core conformation leads to receptor desensitizataion, while tail conformation initiates receptor endocytosis (internalization) and receptor signaling on its own (Cahill et al, 2017). β-arrestin-inactivated receptor is trafficked to early endosomes (Jean-Alphonse et al, 2014) or to very early endosomes (Sposini et al, 2017) in order to be recycled or degraded (**Figure 1.4**). Degradation is

Figure 1.4



GPCR cycle (adapted from Smith et al, Br J Pharmacol **160**, 2010):

Activation of GPCR (green receptor) results in GDP dissociation and replacement with GTP. GRK-mediated phosphorylation is required for β-arrestin binding for receptor desensitization and internalization. Receptor dephosphorylation is followed by either GASP-mediated degradation or recycling.

triggered by binding of GPCR-associated sorting proteins (GASPs) which traffics receptors toward lysosomes (Whistler et al, 2002).

1.3. P1 Receptors

P1 receptors are G-coupled receptors known as adenosine receptors. There are four members in P1 receptors: A_1, A_{2A}, A_{2B}, and A_3 (ADORA1, ADORA2A, ADORA2B, and ADORA3). Upon activation by binding adenosine, ADORA1 induces potassium current through inward potassium channel in cardiac cells resulting in bradycardia (Belardinelli et al, 1995). Activated ADORA2A downregulates migration of leukocytes to inflammation site (Wallace & Linden, 2010). ADORA2B is highly expressed on epithelium during inflammation. It regulates vascular inflammation as it controls epithelial barrier function (Eckle et al, 2008). ADORA3 controls water and chloride transport in epithelial cells of the eye. Therefore, it can be targeted to treat dry-eye syndrome such as with using CF101 (Avni et al, 2010).

1.4. P2 Receptors

1.4.1. P2X Receptors

Activation of P2 receptors mediates intracellular calcium signaling. P2X receptors are ATP-gated ion channels that are sensitive to ATP and, upon activation, are permeable to increase uptake of sodium and calcium coupled with potassium efflux. They form a trimer in a closed mode. Activation causes the subunits to flex and open the channel (Kucenas et al, 2003). There are 7 members in P2X receptors involved in vascular inflammation. For example, activated P2X7 receptors contribute in cytokine release such as secretion of IL-1β by macrophages (Ferrari et al, 1997).

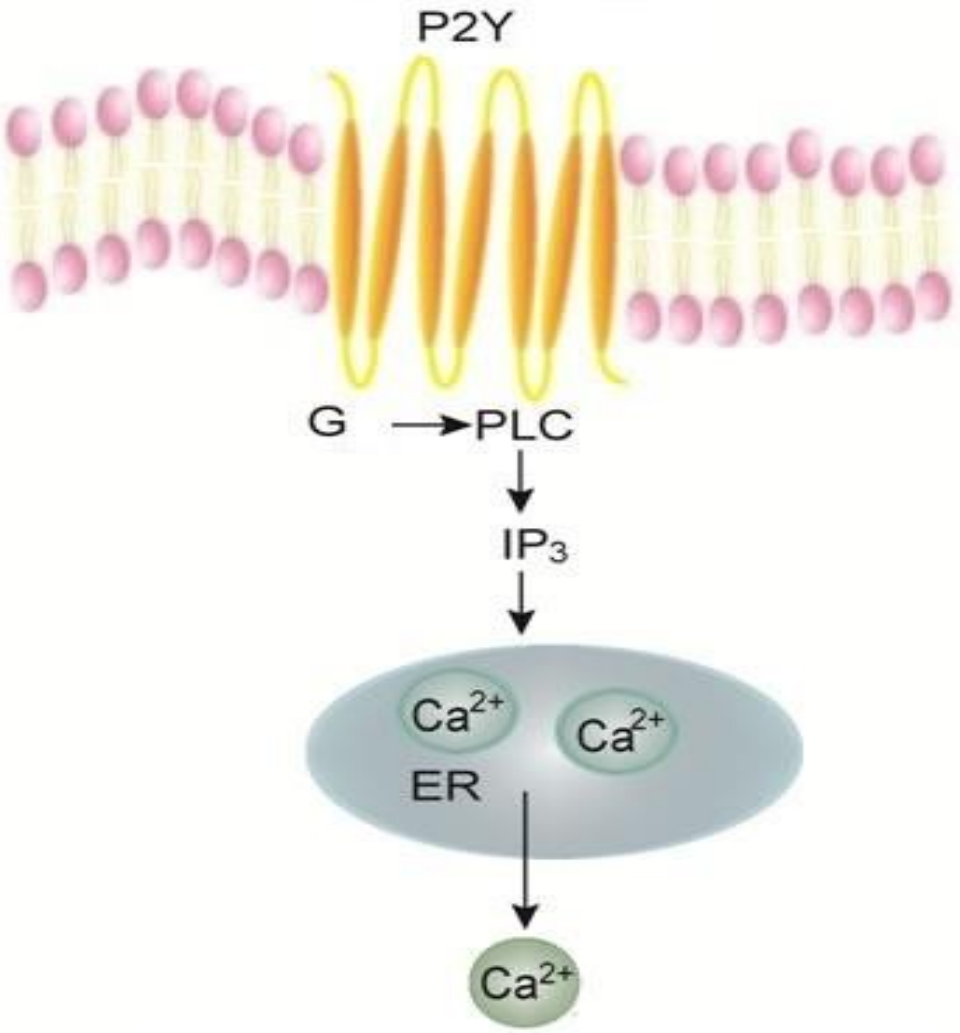
1.4.2. P2Y Receptors

P2Y receptors couple to different G proteins, For example, G_q-coupled P2Y receptors include P2Y-1, 2, 4, 6, and 11. P2Y-12, 13, and 14 can couple to G_i protein. Coupling to G_q protein receptors mediate activation of the receptor-linked phospholipase C (PLC) which breakdowns phosphotidyl inositol 4,5-biphosphate (PIP2) to produce inositol 1,4,5-triphosphate (IP3) and diacylglycerol (**Figure 1.5**). As a result, there will be a release in intracellular $Ca2^+$ stores (Novak, 2003), activation of protein kinase C and protein kinase D (Muscella et al, 2003). PKC phosphorylates kinases upstream of mitogen-activated protein kinases (MAPK). There are three enzymes in the family of MAPK: extracellular signal-regulated kinase (ERK), p38, and c-Jun NH_2-terminal kinase (JNK). ERK activation is also mediated by G_q-activated PKD or G_s-activated PKA. G_iPCR can activate phosphatidylinositol triphosphate kinase (PI3K) which then phosphorylates two enzymes: phosphoinositide-dependent kinase 1 (PDK-1) and mTORC2, both of which are required for a full activation of Akt enzyme. In essence, receptor coupling to G_q can lead to activation of ERK, p38, and JNK signaling pathways and inhibition of Akt signaling pathway independently from PLC activation.

There are 8 members in the P2Y family. In this section, physiological functions for each P2Y receptor will be covered briefly except P2Y2 in more details. P2Y1 is activated by ADP and involved in platelet aggregation through P2X1-mediated activation of phospholipase C and thus increasing intracellular calcium signaling (Hechler et al, 2003; Jones et al, 2014). Platelet activation can also be achieved by ADP-induced P2Y12. Activated P2Y12 is coupled to the Gαi subunit of the G-protein to inhibit adenylyl cyclase that converts ATP to cAMP. Consequently, platelets can be activated by the low concentrations of cAMP. UTP-activated P2Y4 is mainly involved in cardiac injury. It protects the heart from inflammation and fibrosis (Horckmans et al,

2015). Furthermore, it is involved in post-ischemic cardiac remodeling (Horckmans et al, 2012a), and exercise cardiac tolerance (Horckmans et al, 2012b). Additionally, Robaye et al (2003) demonstrated that P2Y4 stimulates water and chloride secretion from intestinal epithelial cells. P2Y6 is activated by UDP and stimulates IL-8 production by eosinophils (Idzko et al, 2003). Activated P2Y6 contributes in pulmonary inflammation by triggering release of IL-8 and macrophage inflammatory protein-1 (MIP-1) from nasal epithelial cells, monocytes, and dendritic cells to promote neutrophils migration to the site of inflammation (Marcet et al, 2007), remodeling of pulmonary inflammatory tissues (Vieira et al, 2011) and of pulmonary fibrosis (Muller et al, 2017). Moreover, it inhibits activation of $CD4^+$ T lymphocytes and therefore protects lungs against allergen-induced pulmonary inflammation (Giannattasio et al, 2011). Furthermore, during allergic inflammation, P2Y6 stimulates degranulation of basophils (Nakano et al, 2017). P2Y11 is activated by ATP and has anti-inflammatory effects in atherosclerosis. Activated P2Y11 limits epithelial cells apoptosis (Urban et al, 2012), reduces epithelial oxidative stress and as a result improves vascular function (Danila et al, 2017). P2Y11 limits Th1 lymphocytes and simultaneously increases Th2 lymphocyte activity (Chadet et al, 2015). ADP-induced P2Y13 receptors are involved in HDL, High Density Lipoprotein, metabolism by increasing hepatobiliary reverse cholesterol transport (Serhan et al, 2013). As a result, activated P2Y13 inhibits atherosclerosis progression (Goffinet et al, 2014) and development (Lichtenstein et al, 2015). Finally, P2Y14 is activated by UDP-glucose and involved in platelet-induced monocyte and neutrophil chemotaxis to platelet-leukocyte junction in pulmonary inflammation (Amison et al, 2017). It mediates vasoconstriction of the porcine coronary artery, which makes it a therapeutic target for ischemic heart disease (Abbas et al, 2017).

Figure 1.5



P2Y receptor-mediated Ca^{2+} signaling (adapted from Wan et al, Oncotarget **7**, 2016):

Stimulated P2Y receptor activates PLC which breaks down PIP2 into IP3 and DAG. IP3 mediates calcium release from endoplasmic reticulum (ER) to cytosol.

1.5. P2Y2 Receptor

P2Y2 is a 377-amino-acid membrane receptor that is ubiquitously expressed on different mammalian cells. It is activated by ATP or UTP nucleotides. P2Y2 receptors play major roles in cellular physiology in different aspects including inflammatory responses and apoptosis such as cytokines production and cellular homeostasis. Based on the class of coupled G protein, P2Y2 activates different pathways. G_q coupled P2Y2 mediates activation of PLC that is involved in inhibition of bone formation and mineralization. G_o-activated PLC is involved in leukocyte recruitment via Rho activation of NF-κB. G_{12} coupled receptor triggers Rho activation and is involved in thrombosis, water/Cl^- secretion in renal homeostasis, and wound healing. Similar to other P2Y receptors, activated P2Y2 receptors signal activation of Src/p38 leading to ERK phosphorylation in coronary artery endothelial cells (Ding et al, 2011). Thus, Src-mediated ERK phosphorylation results in increased VCAM-1 production and more monocyte recruitment (Seye et al, 2003). Also, P2Y2 controls blood vessel permeability and leukocyte trafficking via activation of Rho-GTPase (Rac-1), which mediates homophylic adhesion between vascular endothelial cells by activating vascular endothelial catherin (Liao et al, 2014). Furthermore, stimulated P2Y2 activates phospholipase A_2 which promotes the synthesis of prostaglandins through release of arachidonic acid (Xing et al, 1999; Welch et al, 2003). In cancer progression, platelet ATP-activated P2Y2 receptor induces endothelial permeability leading to migration of tumor cells and cancer metastasis (Schumacher et al, 2013). P2Y2 receptors are involved in improvement in cardiac functions such as protection from hypoxia and post-ischemic myocardial damage (Hochhauser et al, 2013) and reducing infract size (Cohen et al, 2011). They, also, contribute in vasorelaxtation via increasing production of nitric oxide (NO) and cystolic $Ca2^+$ leading to increased membrane hyperpolarization (Raqeeb et al, 2011). In atherosclerosis, P2Y2 initiates

ICAM-1-induced lymphotoxin-α (LTA) production via filamin A (FLN-A) recruitment. FLN-A provides Rho signaling upstream LTA release (Seye et al, 2012). Moreover, FLN-A increases migration of vascular smooth muscle cells to atherosclerotic lesions (Yu et al, 2008) and alters cytoskeletal reorganization in VMSCs leading to increased expression in low-density lipoprotein receptor protein. As a result, there will be an increase in aggregated-LDL uptake and atherosclerosis progression (Dissmore et al, 2016). In P2Y2-deficient mice, reduced LTA levels delayed atherosclerotic onset because of the inhibition in fatty streak formation (Qian et al, 2016; Shaomin et al, 2016). However, there was extensive calcification in atherosclerotic lesions in P2Y2-deficient animal models (Shaomin et al, 2017). Activated P2Y2 receptors results in increased production of VCAM-1 (Seye et al, 2002), MCP-1, and macrophage inflammatory protein-2 leading to increased leukocyte recruitment, rolling, and adhesion with a dominant macrophage content (Stachon et al, 2016). VCAM-1-mediated eosinophil accumulation is induced in allergic pulmonary inflammation (Vanderstocken et al, 2010). Besides inflammatory responses, P2Y2 mediates macrophage recruitment in apoptosis (Elliott et al, 2009) and inhibits neutrophil recruitment by increasing lactferrin production (Bournazou et al, 2009). More physiological functions of activated P2Y2 has been observed in wound healing (Boucher et al, 2010), in self-organization of salivary epithelial cells into acinar-like spheres (El-sayed et al, 2014), and in IL-1-activated mucous oversecretion in airway inflammation (Jeory et al, 2016). Furthermore, P2Y2 contributes in renal water and salt homeostasis in two ways. First, it inhibits epithelial sodium channels and thus increases extracellular fluid (Knepper et al, 2003; Pochynyuk et al, 2010). Second, increased intracellular Ca^{2+} levels activate chloride secretion through Ca-activated Cl^- channels (Rajagopal et al, 2011). Due to broad activities and multiple functionalities of P2Y2 in different cells, it has been a potential therapeutic target for various diseases.

1.6. P2Y2 Agonists

Natural agonists were the first mediator in research used to activate P2Y2 receptors. INS316, developed by Inspire Pharmaceuticals, is a natural UTP that is used as a sputum induction agent in untreated lung cancer patients. UTP-stimulated P2Y2 induces chloride and water secretion and increases cilia beat frequency. Therefore, P2Y2 downstream signaling improves sputum secretion from lower respiratory tract; and that is very crucial in bronchitis and lung cancer diagnosis (Jablons et al, 2001; Johnson et al, 2002). However, natural agonists are prone to hydrolysis by nucleotidases (Picher & Boucher, 1998). Therefore, modifications to improve resistance to hydrolysis are needed. The first documented modification to nucleotides was developed by Goody et al (1972) with a thio substitution at the terminal phosphate group producing UTP-γ-S (uridine 5'-O-3-thiotriphosphate) and ATP-γ-S (adenosine 5'-O-3-thiotriphosphate). Although addition of sulfer improves resistance to ectonucleotidase, these derivatives still lack selectivity to P2Y2 receptors in comparison to UTP-activated P2Y4 receptors and have rapid metabolism. Shaver et al (1997) synthesized 4-substituted uridine 5'-triphosphate which had the same pharmacological effects as UTP with a slower metabolism. Addition of longer or different side chains to the terminal phosphate group yields more selective P2Y2 agonists such as addition of 4-alkyloxyimino to 4-substituted uridine 5'- triphosphate (Jayasekara et al, 2014). Modification of ribose moiety with addition of β, γ difluoromethylene such as in PSB1114-4-thio-uridine shows >60-fold higher selectivity for P2Y2 receptors compared to P2Y4 (El-Tayeb, 2011).

Diquafosol tetrasodium (Santen Pharmaceuticals), known as INS365, was the first P2Y2 agonist approved and launched in 2010 in Japan to treat dry eye syndrome. The addition of di-uridine polyphosphate increases the compound stability and decreases its metabolism. The UTP-derivative INS365, P1,P4 di-uridine-5'-tetraphosphate, improves ocular surface health by

stimulating P2Y2-mediated activation of PLC and IP_3 leading to water secretion via chloride channels, mucin secretion and tear production, and restoring corneal barrier functions (Fujihara et al, 2001). In treatment of cystic fibrosis, INS37217 (P1-(uridine 5')-P4-(2'-deoxycystidine 5') tetraphosphate, tetrasodium salt) is structurally similar to INS365 except replacement of one uridine with deoxycystidine makes INS37217 more resistant to enzymatic hydrolysis. As a UTP-derivative, INS37217 increases chloride and water secretion and mucin release. With the advantage of being resistant to hydrolyzing enzymes, it shows more effective and improved mucociliary clearance, which is a significant advantage in treating cystic fibrosis (Yerxa et al, 2002). MRS2768 (uridine-5'-tetraphosphate σ-phenyl ester) enhances P2Y2-mediated post-ischemic cardioprotection from hypoxia (Hauchhauser et al, 2013).

1.7. P2Y2 Antagonists

Unfortunately, it has been difficult to develop P2Y2 selective antagonists due to their metabolic instability and low selectivity although the significant importance of antagonists as potential therapeutic agents to treat cancer and inflammation. Suramin is an anti-trypanosmiasis used as a broad spectrum competitive antipurinergic drug that inhibits ectonucleotidase (Hourani and Chown, 1989). Suramin does not discriminate between P2X and P2Y receptors (Kennedy et al, 1990). Suramin derivatives, NF 864, NF 449, NF 110, and NF 023, showed selective antagonistic activity against P2X. NF 864 and NF 449 completely block P2X1 and can be further investigated as antithrombotic agents (Horner et al, 2005; Hechler et al, 2005). Another non-selective competitive antagonist that is widely used to inhibit P2 receptors is the anthraquinone derivative Reactive Blue 2 (RB-2) dye. RB-2 dye has a moderate affinity for P2X and P2Y receptors and blocks IP_3 production (Brown and Brown, 2002). There are several derivatives assessed for enhanced selectivity. For example, Uniblue 1 and acid Blue 129 developed by Tuluc

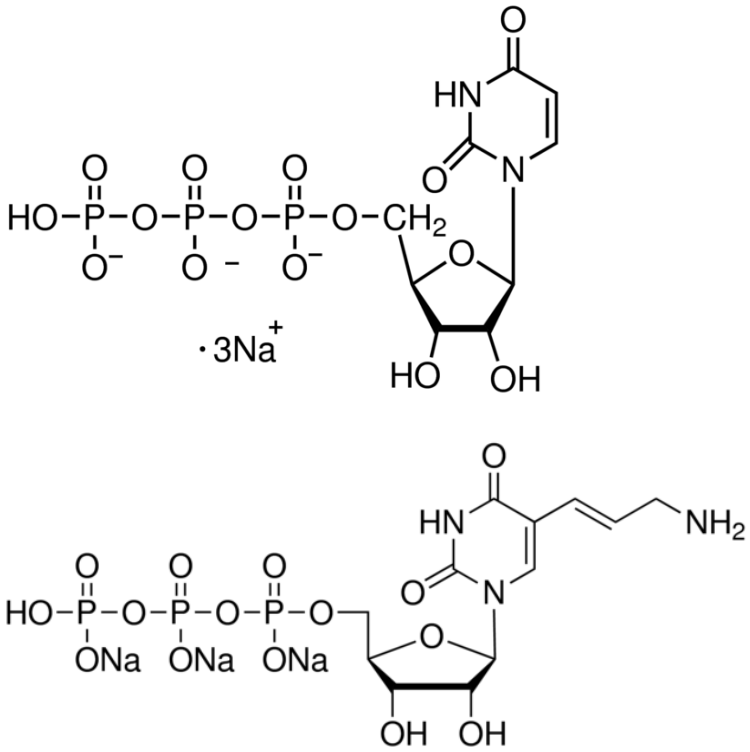
et al (1998) are P2X and P2Y selective, respectively. MG 50-3-1 has more affinity for P2Y1 receptors (Glänzel et al, 2005). Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid, PPAD, is an allosteric antagonist that also reduces IP_3 production (Brown et al, 1997; Vigue et al, 1998). PPAD prevents P2Y-mediated liver cirrhosis by inhibiting hepatic stellate cells proliferation (Dranoff et al, 2007). Also, PPAD mediates reduced production of inflammatory IL-1β, IL-6, and NO (Martucci et al, 2008). LPS-induced neutrophil migration is inhibited by PPAD-mediated P2Y4 antagonism (Kukulski et al, 2009). It is not fully understood how PPAD behaves as an allosteric antagonist. A recent study by Schwiering (2017) focusing on Hemolysin A host toxicity. Hemolysin A is a pore-forming toxin produced by *Staphylococcus aureus*. Hemolysis in experimental erythrocytes is inhibited by P2X inhibitors. However, P2X antagonists surprisingly prevent hemolysis in cells lacking P2X receptors suggesting another P2X-independent mechanism of action of PPAD. With further investigation, PPAD directly binds Hemolysin A.

AR-C118925 is a UTP-analog thiouracil derivative synthesized by replacing ribose triphosphate and derivatizing uracil (Hochhauser et al, 2013). The compound is the only promising P2Y2 antagonist in literature. AR-C118925 (**3**) and its derivatives, **4** and **5**, were resynthesized and evaluated by Rafehi et al (2017). AR-C118925 is a competitive antagonist that blocks P2Y2-receptor-mediated calcium release and inhibits β-arrestin-mediated G-protein translocation required for GPCR recycling. They showed that the modified compound (**3**) is at least 50-fold selective for P2Y2 when compared to other P2Y and P1 receptors. However, there were notable antagonistic activities on P2X1 and P2X3 receptors although selectivity was higher for P2Y2 with 50-fold and 14-fold, respectively.

1.8. Aminoallyl-UTP

Aminoallyl-UTP (**Figure 1.6**) has been used to produce labelled recombinant DNA and RNA for microarrays fluorescence detection. Incorporation of aminoallyl-UTP in a polymerase chain reaction allows coupling with a fluorescence dye in the second round of amplification (Postier et al, 2003; 't Hoen et al, 2003). To our best knowledge, the effect of aminoallyl-UTP on P2Y2 receptor has not been studied and reported yet. Therefore, we assessed the possible modulation role of aminoallyl-UTP on P2Y2 receptors.

Figure 1.6



Chemical Structure of UTP (top) and aminoallyl-UTP (bottom):

Aminoallyl-UTP (PubChem SID 24891009) was developed by the addition of amine group to UTP (PubChem SID 329827645). (Sigma).

Chapter 2. Material and Methods

2.1. Cell Culture:

2.1.1. HeLa cells:

HeLa cells culture was purchased from the American Type Culture Collection (ATCC). HeLa cells were cultured in DMEM supplemented with 10% FBS in a 5% CO_2 humidified atmosphere. Prior to stimulation, cells were seeded in a 10-cm dish and expanded to the appropriate density, starved overnight, and treated with 10% FBS at indicated time and concentration.

2.1.2. Human coronary artery endothelial cells:

Human coronary artery endothelial cells (HCAEC) were purchased form Lonza Group Ltd. HCAEC were cultured in EBM-2 medium supplemented with VEGF, FGF, EGF, IGF, ascorbic acid, GA1000 (Lonza), and 5% FBS at 37°C in a 5% CO_2 humidified atmosphere in Forma Series II Water Jacketed incubator (Thermo). HCAECs between the third and eighth passages were used for experiments. Prior to stimulation, cells were seeded to grow for 24 hours and starved overnight.

2.1.3. P2Y2-transfected 1321N1 astrocytoma cells:

P2Y2R-transfected 1321N1 cell lines were kindly offered by Dr. Gary Weisman (The University of Missouri-Columbia). P2Y2R-transfected 1321N1 cells were cultured in DMEM supplemented with 10% FBS with or without 0.5 mg/mL G418.

2.2. Long-term storage:

For long-term storage, healthy cells with a viability of >90% free of microbial contamination were collected by centrifuge at 1100 rpm for 5 minutes. Cells at concentration of approximately 3 x 10^6 cells/mL were resuspended with cryoprotectant dimethyl sulphoxide (DMSO) at a final concentration of 10% in 2-ml tubes (Greiner Bio One). The vials were first transferred to isopropanol freezing container (VWR) to reach 1°C/min cooling rate and kept at -80°C overnight. The next day, vials were stored in liquid phase nitrogen to ensure the lowest possible storage temperature in order to maintain absolute consistency. Each vial was individually labelled with a cell line's name and the lot number and recorded on an electronic database and a spreadsheet.

2.3. Intracellular Ca^{2+} mobilization assay:

Cells were seeded at a density of 4 x 10^4 cells/well into 96-well culture plates and cultured for one day. On day two, the original medium was removed and replaced with the assay medium from FluoForteTM kit (Enzo Life Sciences) containing the Ca^{2+} dye; and receptor-mediated Ca^{2+} mobilization was determined as previously described (Ding, Ma et al. 2011). Briefly, cells and Ca^{2+} dyes were incubated at 37ºC followed by incubation at room temperature for 45 minutes and 15 minutes, respectively. For antagonist inhibition experiment, cells were incubated first for 30 minutes at 37ºC followed by addition of antagonist and re-incubation for 15 minutes at 37ºC and 15 minutes at room temperature. UTP and aminoallyl-UTP were purchased from Sigma. 20 µL of receptor agonist were added to the mixture; and fluorescence was determined immediately with an excitation wavelength set to 485 nm and an emission wavelength set to 525 nm in the fluorometer plate reader 36 (BMG FLUOstar). The readings were taken every 5 seconds for 500 seconds. The data were shown as relative fluorescence units (RFU) and analyzed by using Prism 5 (Graphpad

Software Inc.). For dose response experiments, the highest RFU was determined; and the RFU of lower doses was calculated accordingly as percentages.

2.4. Western blotting assay.

2.4.1. Sampling

Chemicals and buffers used for Western blot in this study are listed in **Table 2.1**. After stimulation, adherent cells were lysed in Laemmli sample buffer (Sigma-Aldrich) and scratched with cell lifter. Briefly, the cells were first centrifuged; and the cell pellets were resuspended in Laemmli sample buffer. All lysates were collected on ice and then heated in boiling water for 5 minutes.

Table 2.1. List of chemicals used in Western Blotting

Item	Catalog Number	Company
10X Tris-Glycine	75894	Affymetrix-USB
10 X Tris-Glycine-SDS	97062-364	VWR Life Science
10X TBS	J60764	Alfa Aesar
Tween20	P7949	Sigma
Non-fat dry milk	170-6404	Bio-Rad

Electrophoresis buffer was prepared with 25mM Tris, 192 mM Glycine and 0.1 % (w/v) SDS at pH 8.3. Transfer buffer contains 25mN Tris, 192mM Glycine with 20% (v/v) methanol at pH 8.3 Tris-Buffered Saline and Tween 20 were made from 10X TBS (pH 7.4) containing 25 mM Tris, 2.6 mM KCl, 137 mM NaCl, and 1.0% Tween-20 working concentrations. Blocking buffer

was made with 5% non-fat dry milk in 1X TBST. Primary antibody was diluted in 5% non-fat milk in TBS.

2.4.2. Blotting

Samples were loaded and separated on 10% Mimi-Protean® TGXTM Precast Gel (Bio-Rad) in a SDS-PAGE gel chamber (Bio-Rad) filled with electrophoresis buffer for 45 min with the voltage of 110V. The gel was placed in between two transfer buffer pre-soaked filter papers and polyvinylidene fifluoride (PVDF) membrane (Thermo) using semi-dry blotting apparatus (Bio-Rad) for 35 minutes with the voltage of 20V. After transfer, the membrane was blocked with 5% fat-free milk in TBST for 1h (room temperature) on shaking rocker rotating at 60 rpm. The membrane was washed with TBST for 4 times, 10 minutes each. Then, the membrane was probed with the primary antibody and incubated overnight at 4^{o}C in 1X TBST containing 5% BSA with gentle shaking. The antibodies used for Western in this study are listed in **Table 2.2**. On the following day, the membrane was washed with TBST 4 times, 10 minutes each. The secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (Cell Signaling Technology Inc.) diluted in 1X TBST containing 5% non-fat dry milk was added and incubated for 1 h at room temperature. Unbound antibodies were washed out with TBST for 4 times, 10 minutes each. The membrane was incubated briefly for 2 minutes with Western Lightning® Plus-ECL(PerkinElmer).

Table 2.2. The antibodies used in Western Blotting:

Items	Catalog Number	Company
p-ERK	4370T	Cell Signaling
t-ERK	4695T	Cell Signaling
p-p38	4511T	Cell Signaling
t-P38	8690T	Cell Signaling
p- Akt-S473	4066T	Cell Signaling
t- Akt-S473	4691T	Cell Signaling
p- Akt-T308	13038S	Cell Signaling
t- Akt-T308	4691T	Cell Signaling
p-JNK	4668T	Cell Signaling
t-JNK	9252S	Cell Signaling
Anti-rabbit IgG	7074S	Cell Signaling

2.4.3. Imaging analysis

The membrane image was developed on a photographic film (Research products international corp.) and visualized by medical film processor (Konica Minolta Medical &graphic Inc). The image was scanned by greyscale and converted to a digital image. The intensity of signals was analyzed with densitometric software, Quantity One. Data were normalized against the control background and given in a relative density percentage.

2.4.4. Stripping and re-probing

In order to remove bound primary and secondary antibodies (stripping) and to re-probe proteins with another set of antibodies, blots were first washed with TBST and then incubated with

Restore PLUS Western blot stripping buffer (Thermo) for 20 minutes at room temperature. The membrane was washed with TBST for 30 seconds and blocked at room temperature with 5% fat-free milk in TBST for 1 hour with shaking at 60 rpm. The proteins are re-probed with desired antibodies.

Chapter 3. Results

3.1. Evidence of cell-specific aminoallyl-UTP mediated Ca^{2+} signaling:

In order to demonstrate the activity of aminoallyl-UTP on cell receptors, Ca^{2+} mobilization assay was performed in HeLa, HCAEC, and hP2Y2R-1321N1 cells treated with different concentrations of aminoallyl-UTP.

3.1.1. HeLa Cells:

Figure 3.1A shows that aminoallyl-UTP does not mediate Ca^{2+} signal in the HeLa cells treated with 10 and 100 µM aminoallyl-UTP. Using 100 µM aminoallyl-UTP did not have a noticeable antagonistic activity since 10 and 1 µM UTP efficiently mediated an intracellular Ca^{2+} signal (**Figure 3.1B & C**).

3.1.2. HCAEC:

Two HCAEC lines were treated with 10 and 100 µM aminoallyl-UTP separately. Ca^{2+} signaling was measured and compared to UTP-treated cells. Aminoallyl-UTP did not have any effect on Ca^{2+} mobilization in HCAEC (**Figure 3.2A**). 1 µM UTP was able to initiate Ca^{2+} signaling in cells pre-treated with 100 µM aminoallyl-UTP (**Figure 3.2B**).

3.1.3. hP2Y2R-1321N1:

hP2Y2-1321N1 cells were treated with 100 µM aminoallyl-UTP and compared to 100 µM UTP. 100 µM aminoallyl-UTP showed a full agonistic activity significantly similar to UTP on P2Y2 receptor (**Figure 3.3**). A UTP and AU dose-response assays were performed on hP2Y2R-

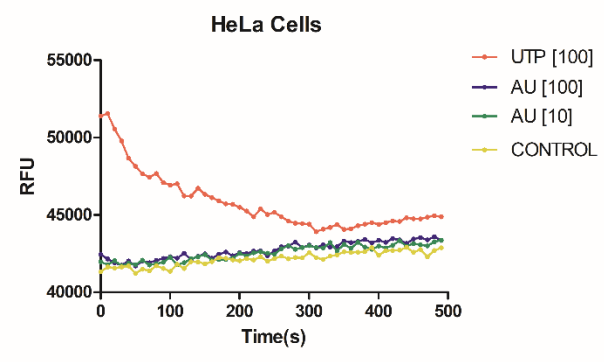
1321 cells. UTP had a dose-dependent activity on P2Y2 receptor, with EC_{50} value being at 3.23 X 10^{-3} µM. On the other hand, EC_{50} value of aminoallyl-UTP was 19.77 µM (**Figure 3.4)**. Aminoallyl-UTP was used as an antagonist. Figure 3.5A shows that 100 µM aminoallyl-UTP can completely desensitized P2Y2 receptor nearly to base line of untreated cells; and partially inhibited 1 µM-UTP-induced Ca^{2+} signal by %50. On the other hand, when cells were pre-treated with 10 µM aminoallyl-UTP and then activated with 0.1 µM UTP, they had a Ca^{2+} signaling similar to AU^{-}/UTP^{+} cells (**Figure 3.5B**).

3.2. Evidence of aminoallyl-UTP mediated activation of Akt signaling pathway in HCAEC:

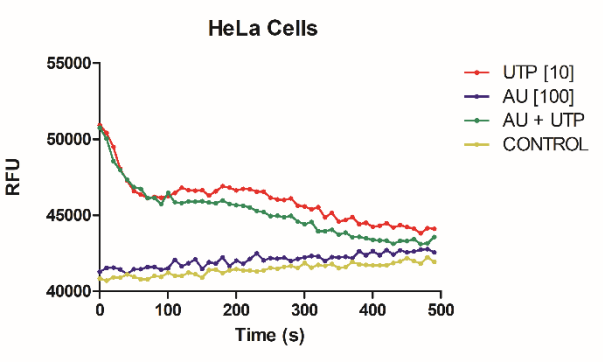
Phosphorylations of MAPK and Akt signaling pathways in aminoallyl-UTP-treated HCAEC were determined with a western blot. Cells were treated with 0.1, 1, 10, and 100 µM aminoallyl-UTP and compared to 100 µM UTP treatment. ERK, p38, and JNK signaling pathways were activated by UTP, but not by aminoallyl-UTP. Surprisingly, among all concentrations including 100 µM aminoallyl-UTP, only 10 µM aminoallyl-UTP mediated Akt phosphorylation (**Figure 3.6 & 3.7**).

Figure 3.1

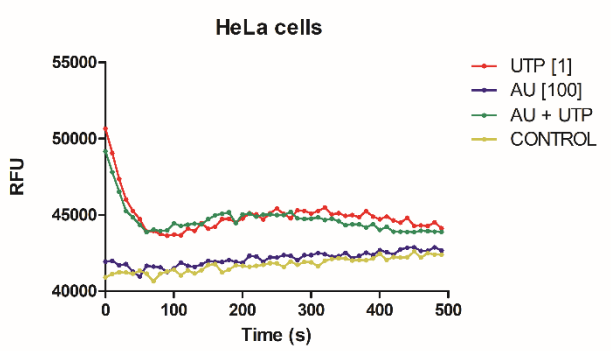
A



B



C

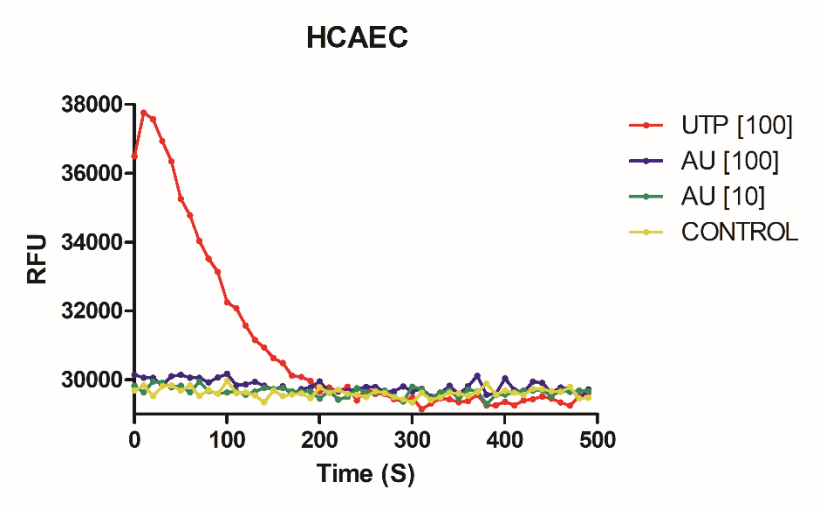


Evidence of Hela Cells Resistance to Aminoallyl-UTP- mediated Ca^{2+} Signaling:

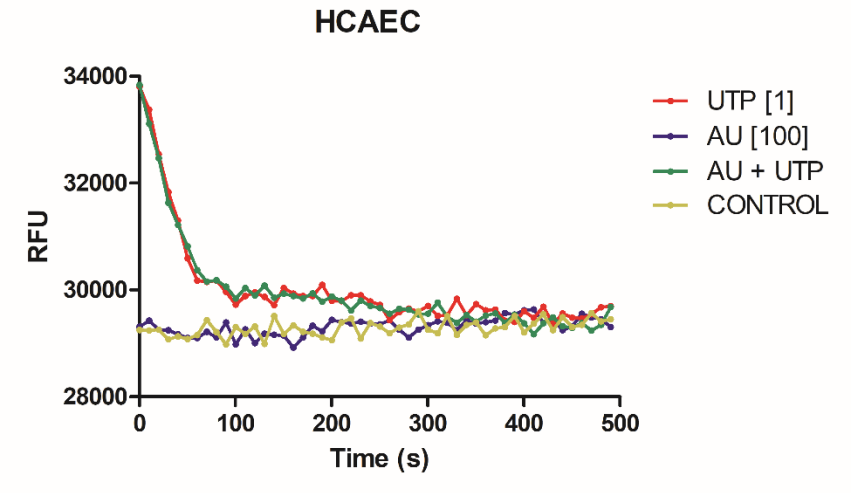
Ca^{2+} mobilizations were not mediated in HeLa cells treated with 10 and 100 µM aminoallyl-UTP, while 100 µM UTP efficiently mediated Ca^{2+} signal (**A**). 100 µM Aminoallyl-UTP did not antagonize the effect of 10 or 1 µM UTP (**B &C**).

Figure 3.2

A



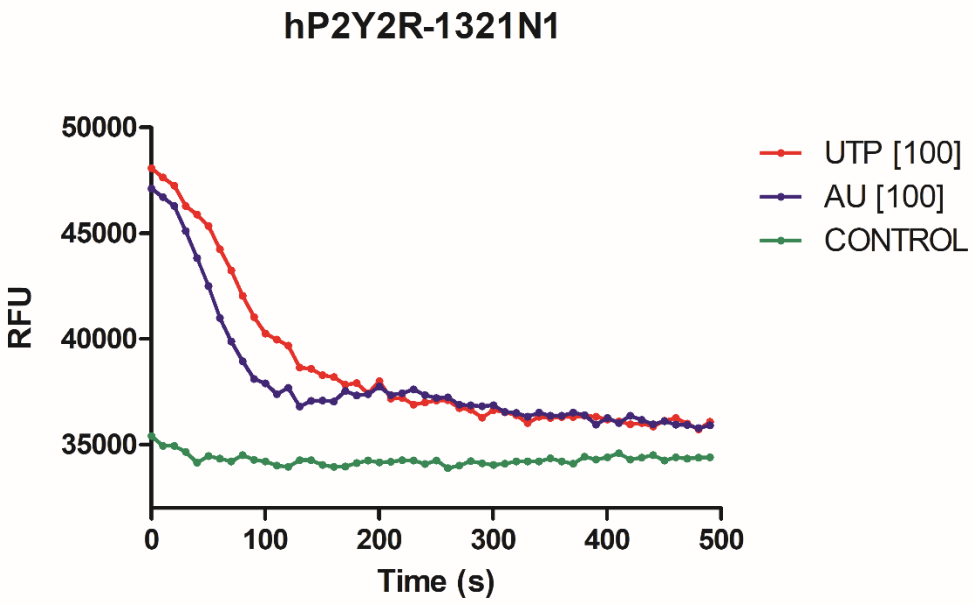
B



Evidence of HCAEC Resistance to Aminoallyl-UTP- mediated Ca^{2+} Signaling:

Ca^{2+} mobilizations were not mediated in HCAEC treated with 10 and 100 µM aminoallyl-UTP, while 100 µM UTP efficiently mediated Ca^{2+} signal (**A**). 100 µM Aminoallyl-UTP did not antagonize the effect of 1 µM UTP (**B**).

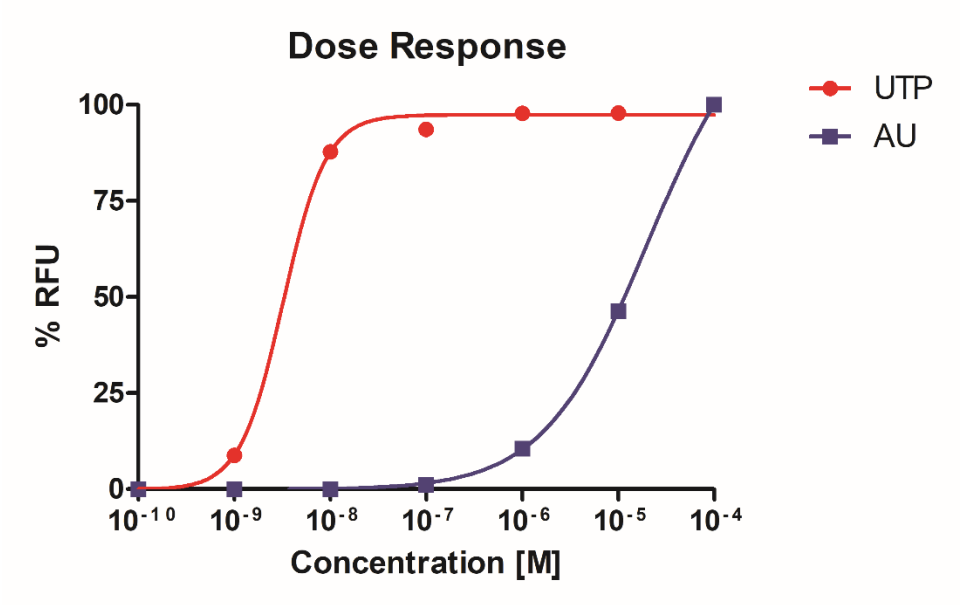
Figure 3.3



Evidence of aminoallyl-UTP-mediated Ca^{2+} signaling in P2Y2R-transfected 1321N1 astrocytoma cells:

100 µM aminoallyl-UTP had an efficient full agonistic activity and mediated Ca^{2+} signal when compared to 100 µM UTP with minor difference.

Figure 3.4

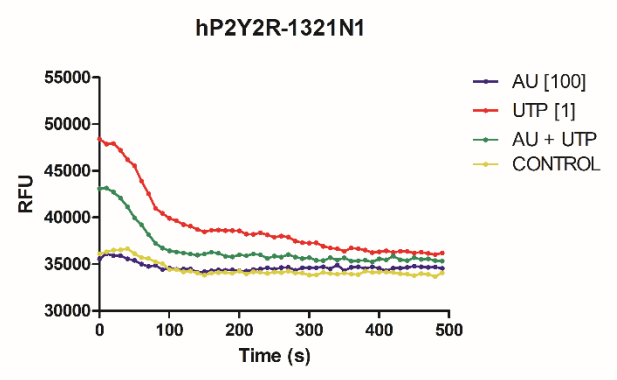


Dose response of UTP and aminoallyl-UTP on P2Y2R-transfected 1321N1 astrocytoma cells:

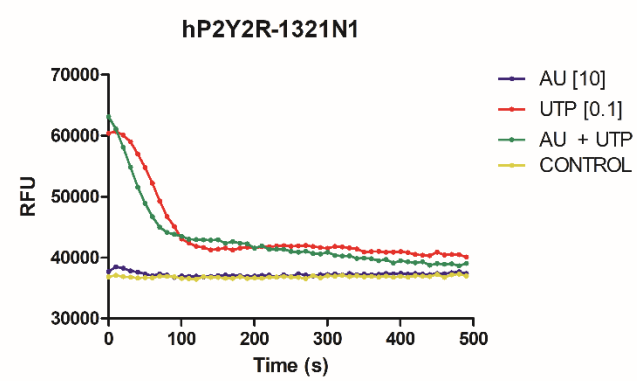
Although both compounds have the same efficacy to P2Y2 receptor at concentrations of 10^{-4} M, EC_{50} value of UTP is about 10,000-fold lower than aminoallyl-UTP.

Figure 3.5

A



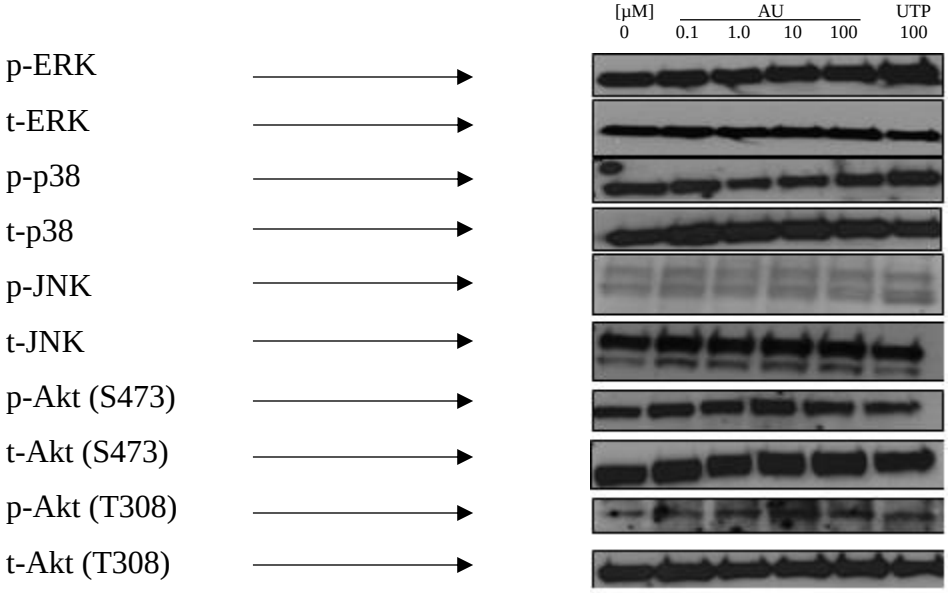
B



Evidence of aminoallyl-UTP mediated desensitization of P2Y2 receptor in P2Y2R-transfected 1321N1 astrocytoma cells:

100 µM aminoallyl-UTP desensitized P2Y2 receptor and therefore reduced UTP-mediated Ca^{2+} signal (**A**). 10 µM aminoallyl-UTP may desensitize less P2Y2 receptor, and thus with no effect on UTP-mediated Ca^{2+} signal (**B**).

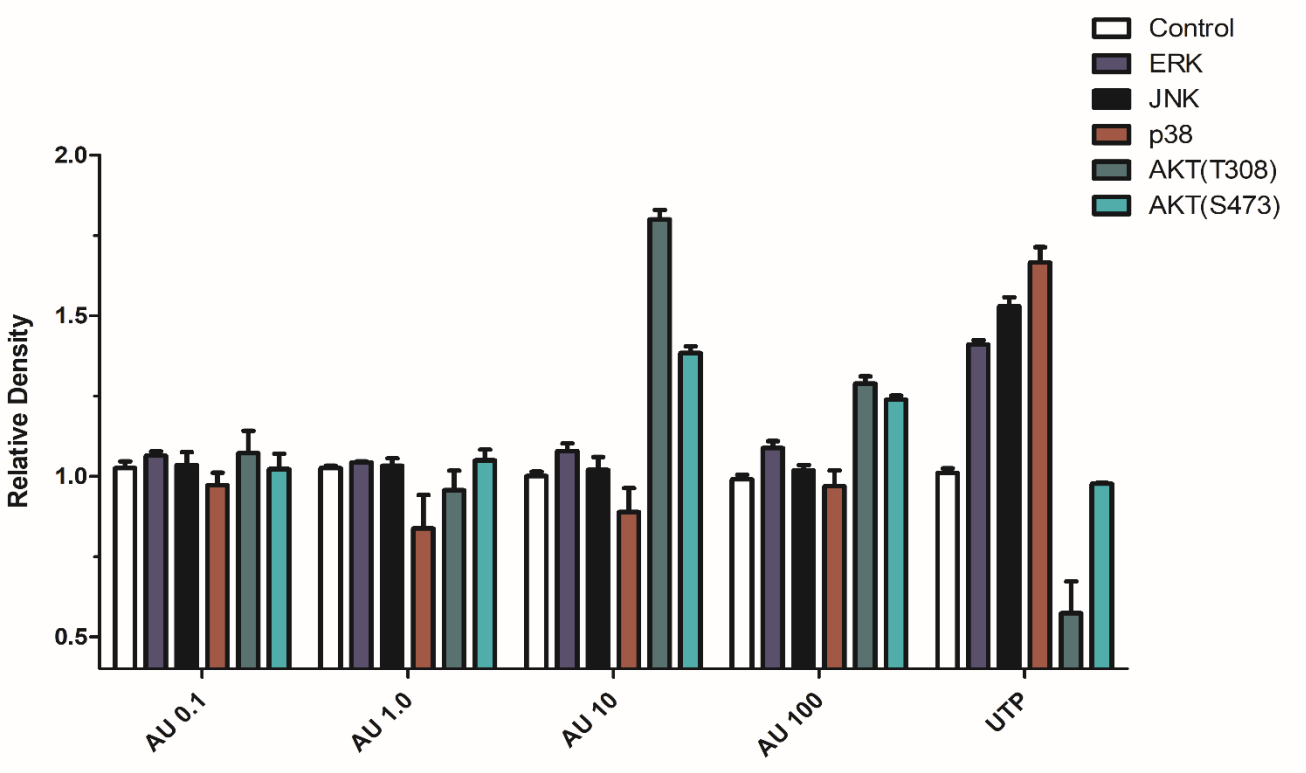
Figure 3.6



Effects of UTP and aminoallyl-UTP on P2Y2R-mediated signaling pathways activation in HCAEC:

100 µM UTP mediated activation of MAPK pathways; and it has inhibitory effect on Akt pathway. However, aminoallyl-UTP did not mediate MAPK phosphorylation. Only 10 µM of aminoallyl-UTP had a stimulatory effect on Akt activation.

Figure 3.7



Densitometric analysis of western blotting bands:

100 µM UTP mediated activation of MAPK pathways; and it has inhibitory effect on Akt pathway. However, aminoallyl-UTP did not mediate MAPK phosphorylation. Only 10 µM of aminoallyl-UTP had a stimulatory effect on Akt activation. The experiment was done in triplicate; data were normalized to control values; and mean was calculated and blotted.

Chapter 4. Discussion

UTP, as well as ATP, is the natural agonist to stimulate P2Y2 receptor. Several modifications of UTP have been developed in order to increase the agonistic selectivity and stability. Here, we observed that aminoallyl-UTP is a cell-specific biased ligand. Aminoallyl-UTP mediated Ca^{2+} signaling only in hP2Y2-transfected 1321N1 astrocytoma cells in a dose-dependent manner. Aminoallyl-UTP-activated P2Y2 receptors were seen to induce phosphorylation of Akt signaling pathway, but with no effect on MAPK pathways in human coronary artery endothelial cells.

Although aminoallyl-UTP has the same efficacy as UTP to P2Y2 receptor in transfected 1321N1 astrocytoma cells, UTP was observed to be more robust in Ca^{2+} signaling in P2Y2R-transfected 1321N1, HeLa, and HCAEC. There are two possible reasons for the cell-specific biased agonistic activity of aminoallyl-UTP. First, aminoallyl-UTP might not have bound to G_q-coupled receptors in HeLa cells and HCAEC. This is accompanied with the membrane structure and environment of HeLa cells and HCAEC. We can hypothesize that these cells might have unique proteins that interact with P2Y receptors to make the receptors more selective to UTP and cannot tolerate even minor modifications in the agonist structure. Therefore, aminoallyl-UTP was not able to bind G_q-coupled P2Y2 receptors and did not mediate Ca^{2+} signaling. Second, hP2Y2-transfected 1321N1 cells have a higher receptor density. Therefore, the exogenously receptor transfection leads to higher agonistic sensitivity.

Aminoallyl-UTP activates P2Y2 receptors in transfected 1321N1 astrocytoma cells with the same efficacy as UTP at concentrations of 100 µM. However, the potency of aminoallyl-UTP is 10,000-fold lower than UTP. When aminoallyl-UTP was used to antagonize UTP-mediated Ca^{2+} signaling, pre-treatment with 100 µM aminoallyl-UTP showed decreased signals of 1 µM UTP activation although 100 µM aminoallyl-UTP completely desensitized the receptor close to basal line of negative control. Aminoallyl-UTP might not have utilized all available receptors; and this could have left some reserved receptors for UTP activation. Another explanation of the partial agonistic activity of UTP is post-desensitization receptor recycling leading to receptor translocation on the cell membrane. On the other hand, 10 µM pre-treatment did not affect UTP-mediated Ca^{2+} signals although it was seen to desensitize bound receptors. This can be caused by the lower potency of aminoallyl-UTP on P2Y2 receptors. 10 µM aminoallyl-UTP had 55% lower activity on P2Y2 receptors than 100 µM leaving reserved receptors that were available for UTP binding.

On activated HCAEC, we demonstrated that aminoallyl-UTP mediated phosphorylation of the Akt signaling pathway. MAPK phosphorylations, however, were not activated by aminoallyl-UTP treatment. In contrast, UTP treatment coupled to MAPK phosphorylation but not Akt. Indeed, UTP shows inhibitory effect on Akt signaling pathways in case of G_q protein coupling, but not $G_{i/o}$, independently of PLC activation. UTP- and aminoallyl-UTP-bound P2Y2 receptors may have different configuration structures which can cause coupling to different effectors and signaling proteins. In this case, aminoallyl-UTP might not have mediated coupling to G_q protein due to the different configurational structure. Surprisingly, only 10 µM aminaoally-UTP mediated Akt phosphorylations at S473 and T308. This biased activation is coupled with UTP inhibitory effect on Akt signaling pathway. We can hypothesize that the 3-dimentional structure of aminoallyl-UTP

couples to unique proteins upstream of Akt signaling pathways leading to stimulatory effect of aminoallyl-UTP on Akt and with no effect on ERK, p38, or JNK signaling pathways.

During vascular inflammation, endothelial cells dysfunction in apoptosis; and that contributes to inflammation progression. Akt signaling pathway mediates pro-survival and anti-apoptotic signal transduction in vascular endothelial cells. Therefore, aminoallyl-UTP can be used as a potential endothelial protective compound via activating Akt-mediated anti-inflammatory responses in vascular endothelial cells.

With the addition of the terminal aliphatic amine group, aminoallyl-UTP can be attached to a fluorescent dye and manipulated in post-labeling of nucleic acids in microarrays. Knowing that aminoallyl-UTP binds P2Y2 receptor with the same efficacy as UTP, labeling aminoallyl-UTP with fluorescence can also be used as a bridge to study the P2Y2 receptor in different aspects such as kinetic studies.

In summary, we demonstrated that aminoallyl-UTP is a new P2Y2 receptor ligand, which shows cell-selective and pathway-selective biased signaling property. Our studies provided new evidence that the P2Y2 receptor can be pharmacologically manipulated to target desired signaling pathway in a cell-specific manner.

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