Role of P2 Receptor Expression in ATP-Induced Purinergic Signaling and Cell Migration in Human Hepatocellular Carcinoma Cells

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ABSTRACT

Hepatitis C virus (HCV), a foremost public health issue, is associated with number of pathological disorders naming chronic hepatitis, cirrhosis, and fibrosis and hepatocellular carcinoma. ATP acts as a ligand for P2X receptors that are non-selective ligand-gated ion channels. P2X4 and P2X7 are the most widely expressed proteins in the liver besides other P2X receptor proteins. The basic notion behind this study was to find out the association of prevalence of purinoceptors in hepatitis C virus (HCV) and non-HCV hepatocellular carcinoma (HCC). Immunohistochemistry was used to study the expression of P2X4 and P2X7 receptors on ex-planted liver tissue samples obtained from hepatocellular carcinoma patients. There was a significant increase in P2X4 receptor expression in HCV HCC as compared to non-HCV HCV. But P2X7 receptor expression was not increased in both HCV HCC and non-HCV HCC. Docking was used to study protein-protein interactions (PPIs) of P2X4 and P2X7 receptor protein with HCV envelops protein E1. We found out few interacting residues between HCV E1 and P2X4R and P2X7R.The current study was designed to study the GPCR-associated calcium signaling with mutated downstream signaling proteins. In the next part of the study quantitative modeling of the GPCR-associated signaling was done and specific agonist and antagonist were used to activate and inhibit the calcium signaling. Moreover, we examined the P2 receptor for ATP-induced Ca^{2+} signaling in human hepatocellular carcinoma (HCC) cells. Fura-2-based measurements of the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) exhibited an ATP induced elevation of $[Ca^{2+}]_i$ in*Huh*-7 and HepG2 cells. NF546, a $P2Y_{11}$ receptor agonist was equally effective in elevation of $[Ca^{2+}]_i$ concentration. Whereas, agonists for the P2X receptors (αβmeATP and BzATP), $P2Y_1$ receptor (MRS2365) or $P2Y_2$

receptor (MRS2768) were ineffective. In addition, ATP/NF546-induced increase in the $[Ca^{2+}]_i$ were strongly inhibited by treatment with NF340, a $P2Y_{11}$ receptor antagonist. Trans-well cell migration assay demonstrated that ATP and NF546 induced concentration-dependent stimulation of Huh-7 cell migration. Treatment with NF340 prevented ATP-induced mediated cell migration. Taken together, our results show critical role of P2Y11 in mediating ATP-inducing Ca^{2+} signaling and regulating cell migration in human HCC cells.

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GLOSSARY OF ABBREVIATIONS

A number of abbreviations have been used in this study. The abbreviations are given as:

HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
PLC	Phospholipase C
ER	Endoplasmic reticulum adenine-rich
ATP	Adenosine 5'-triphosphate
GPCRs	G-protein Coupled Receptors
GEF	Guanine nucleotide exchange factors
PI3Ks	Phosphatidylinositol 3-kinases
IP3	Inositol tris-phosphate
RTKs	Receptor tyrosine kinases
KSCs	Kupffer cells
LSEC	Liver sinusoidal endothelial cells
HSCs	Hepatic Stellate cells
HAV	Hepatitis A virus
HBV	Hepatitis B virus
NANBH	Non-A, non- B hepatitis
UTRs	Untranslated regions
TM	Transmembrane
α,β-meATP	α,β-methyleneATP
PTX	Pertussis toxin
FFPE	Formalin fixed paraffin embedded
H2O2	Hydrogen peroxide

RMSD	Root mean square distance
HADDOCK	High Ambiguity driven biomolecular Docking webserver
CPORT	Consensus Prediction Of interface Residues in Transient Complexes
SBS	Standard buffer solution
DMEM	Dulbecco Modified Eagle's Medium
BRN	Biological Regulatory Network
S.E.M.	Standard error of mean
PBMCs	Peripheral blood mononuclear cells
DOPE	Discrete optimized protein energy
($[Ca^{2+}]_i$)	Intracellular Ca^{2+} concentration
BzATP	2',3'-O-(4-benzoylbenzoyl)-ATP
α β meATP	αβmethyleneATP
NS	Non-structural
PPI	Protein-protein interaction
Kb	kilo bases
TME	Tumor microenvironment
PPADS	Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium
IC50	Inhibitory concentration
CNS	Central Nervous system
PBS	Phosphate buffer saline
HRP	Horse reddish peroxidase
DAB	3,3′-Diaminobenzidine
Huh	Human hepatoma

HEK	Human Embryonic kidney cells
DMSO	Dimethyl sulfoxide
PA	Pluronic Acid
DAPI	4',6-diamidino-2-phenylindole
UV	Ultraviolet
PI	Propodium Iodide

Chapter 1

INTRODUCTION

HCV is a member of Flaviviridae family. It is a single-strand RNA virus that is translated into a single polyprotein. This polyprotein is further processed to structural (Core, E1, E2 and P7) and non-structural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins. HCV is one of the major etiological agent of chronic hepatitis, liver fibrosis, cirrhosis and HCC. E1 is among the structural proteins of HCV and have found to play the primary role in viral entry. E1 is a transmembrane glycoprotein having a C-terminal domain responsible for membrane association and membrane permeability changes (Ciccaglione *et al*., 2001). HCV E1 is a fusogenic subunit of envelope protein. It contains 4-5 N-linked glycans and are responsible for virus interaction with cellular receptors (Knipe *et al*., 2007). Hence, viral entry receptors are important to target to stop virus access into the cells (Baldick *et al*., 2010).

Hepatocellular carcinoma (HCC) is the primary liver cancer. Poor prognosis and ineffective treatment of HCC with currently available anti-cancer treatments have made it alarming and a reason for mortality that have come up with the 5-year survival rate being less than 15% (El–Serag & Rudolph, 2007; Emmett *et al*., 2008; Venook *et al*,. 2010). Mammalian cells express numerous both types of P2 receptors. All these

receptors are present in a cell type-specific manner (North & Surprenant, 2000; Ralevic & Burnstock, 1998). Adenosine triphosphate (ATP) has a well-established role in interaction with P2X receptors and P2Y receptors on the cell surface. This will help to induce autocrine and paracrine signaling with variety of physiological functions and pathological processes, including cancers (Adinolfi *et al*., 2012; Virgilio, 2012; Roger *et al*., 2015). ATP acts as an extracellular messenger molecule and it is effective in nano molar concentrations (M) extracellularly in resting cells (Di Virgilio & Adinolfi, 2016). HCV E1E2 proteins are found to raise the levels of different variants of P2XR as studied in huh-7 cells (Manzoor *et al*., 2011).

There has been supporting evidence that in viral RNA-replicating cells, ATP levels are elevated exhibiting HCV RNA replication with decreased cytoplasmic levels of ATP (Ando *et al*., 2012).

The microenvironments in tumour tissues is highly hypoxic that is found to have highest release of ATP (Bodin & Burnstock, 2001; Falzoni *et al*., 2013; Pellegatti *et al*., 2008). A number of contemporary studies of numerous types of cancer cells have shown ATP-induced purinergic signaling. This signaling is very important in terms of controlling cancer cell migration, proliferation and survival via the P2X7 receptor (Adinolfi *et al*., 2015) or $P2Y_2$ receptor (Giannuzzo *et al*., 2015).There have been

reports to indicate mRNA and/or protein expression of the $P2Y_1$ and $P2Y_2$ receptors in primary and immortalized human normal hepatocytes, primary human HCC cells and immortalized human HCC cells (e.g., Huh-7, HepG2 and BEL-7404)(Emmett *et al*,. 2008) and the P2X4 and P2X7 receptors in HepG2 cells, rat and mouse hepatocytes and rat HCC cells (Emmett *et al*., 2008). Further studies demonstrated that activation of the $P2Y_2$ receptor leads to ATP-induced elevation of $[Ca^{2+}]_i$ in human normal liver and human hepatic cancer cells (Schöfl *et al*., 1999; Xie *et al*., 2014).

GPCR are G-coupled protein, mostly abundant in mammalian cells having complex mechanisms for cellular signaling and communication. GPCRs remain mostly inactive on the cell and upon ligand binding cause activation of GPCR through stimulation of exchange of GDP for GTP on the α-subunit (Black *et al*., 2016). These activation signals turn on the downstream signaling switch , such as adenylyl cyclase or phospholipase C, ultimately increasing intracellular second messengers, such as cyclic AMP (cAMP) or inositol tris-phosphate (IP3) (Oldham & Hamm, 2008).

Activation of class IA phosphatidylinositol 3-kinases (PI3Ks) occurs through stimulation of receptor tyrosine kinases (RTKs) and the concomitant assembly of receptor–PI3K complexes. PIP3 serves as a second messenger that helps to activate AKT. Through phosphorylation, activated AKT mediates the activation and inhibition of several targets,

resulting in cellular growth, survival and proliferation through various mechanisms (Vivanco & Sawyers, 2002). The secondary messenger calcium plays key role in regulating cellular processes and hence cellular calcium homeostasis is crucial. In cancer cells, the intracellular calcium concentration ($[Ca^{2+}]i$) is in prominence as it modulates the apoptotic or proliferative pathways of the cell (Satheesh & Büsselberg, 2015). Aabnormal expression of TP53gene and overexpression of MDM2, known to be a negative regulator of p53, are main observant of the cancers. The MDM2-p53 feedback loop plays an important role in tumor progression (Meng *et al*., 2014).

In our previous studies by (Ashraf *et al*., 2013) we have verified the antiviral role of P2X7 in peripheral blood mononuclear cells (PBMCs). Similarly, in another study by (Manzoor *et al*., 2011) P2X4 receptor known to have pro viral role in Huh-7 cells stably transfected with E1E2. To further confirm this antiviral and pro viral role of P2X4 and P2X7 receptors role we decided to use the ex-planted liver tissue samples from HCV and non-HCV induced HCC. Furthermore, protein-protein interaction (PPI) studies of P2X4 receptor protein with viral hepatitis envelop protein E1 will help get an insight of viral entry into the host cells. It is supposed to bring hepatocytes under stressful conditions with massive release of ATP at injury site. We will examine the P2 receptors by inducing ATP-based Ca^{2+} signaling in human HCC. Agonist and

antagonist specific for the receptors will be used to measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using fura-2 for ratio metric measurement. In addition, we will find out the strong increase or up-regulation of specific P2 receptor in human HCC tissues. Formal modeling dynamics will help us in studying the abnormal behavior of proteins in hepatocellular carcinoma. These proteins will be set as discrete states and trajectories. In the next part of the study quantitative modeling of the GPCR-associated signaling will be done and simulation results will be obtained.

Chapter 2

REVIEW OF LITERATURE

2.1 HCV Infection and Pathogenesis

Hepatitis C virus (HCV) infection was found to be associated with a number of pathologies that can range from liver inflammation to hepatic fibrosis and hepatocellular carcinoma with worse pathology leading to liver failure (Choi & Ou, 2006; Sheikh *et al*., 2008; Walters *et al*., 2009). There were cases found to be associated with mild disorders with nonspecific symptoms such as fatigue with accompanied severe complications of cirrhosis with death that might occurred in 15 to 20 percent of those infected. Increased oxidative stress was a main culprit in the development and the progression of HCV induced pathogenesis (Choi & Ou, 2006; Lonrdo, *et al*., 2004; Okuda *et al*., 2002). HCV has been viewed as a true metabolic syndrome rather than a simple viral infection after an emerging insight into the pathogenic mechanism involved in liver fibrosis and its progression. (Kumar *et al.*, 1992; Qadri *et al*., 2004; Akbar, *et al*., 2009; Bataller & Brenner, 2005; Sheikh, *et al*., 2008).

2.2 Purinergic Signaling

Purinergic signaling was first discovered in 1972 with a novel notion where adenosine 5′-triphosphate (ATP) and adenosine acted as extracellular signaling molecules. With the passage of time, more receptors for purines and pyrimidines were cloned and characterized on the basis of their presence on different organs and functions. This discovery led to identification of four adenosine receptors, seven subtypes of P2X ion channel receptors and eight subtypes of P2Y receptors. Initially, these receptors were identified in neuronal as well non-neuronal cells with their physiological role well

characterized. Now these receptors have been studied in different patho physiological conditions of different diseases (Burnstock & Di Virgilio, 2013).

2.3 Purinergic Receptors in the control of Liver Homeostasis

It was well established at the moment that ATP and adenosine were potent signaling molecules similar to hormones, neurotransmitters and other signaling moieties in the body. A number of studies were documented to support the role of ATP-induced signaling in tissue homeostasis. There were various membrane transporters on hepatic cells that could contribute to the purinergic salvage pathway. When there was release of un-metabolized ATP and nucleotides they ultimately supported binding to purinergic receptors (P2X) and subsequently activated downstream signaling molecules conclusively termed as purinergic signaling which have been found to regulate liver glucose metabolism, lipid metabolism, protein synthesis, ion secretion and homeostatic processes like cell cycle and immune responses in liver cells (Fausther *et al*., 2012).

2.4 Purinergic signaling and HCV induced Liver Cancer

HCV perturbed a number of molecular signaling pathways that led to different liver pathologies naming a few liver steatosis, fibrosis, cirrhosis and Hepatocellular carcinoma. There was still need to explore the secondary signal molecules that hampered the normal liver functioning. Adenosine 5′-triphosphate (ATP) was found to be a potent autocrine/paracrine signaling molecule in hepatocytes. Under pathophysiological conditions in the liver like oxidative stress, cell volume changes and inflammation, ATP was released in extracellular spaces of the cells. This ATP acted as a ligand to different purinergic receptor subtypes to activate the downstream calcium signaling and other processes like cell proliferation, apoptosis, fibrosis and

cancer. There are numerous variants of P2X receptors were found to increase in the presence of HCV E1E2 in human hepatoma cell lines (Manzoor *et al*., 2011).

2.5 Purinergic Signaling in Liver Diseases

Purinergic signaling was first discovered in 1970s in cellular systems. There have been two types of receptors adenosine receptors termed as A1 and ATP (P2 receptors) that were activated by extracellular nucleotides and nucleosides played a key role in different aspects of health and diseases involving vital organ of the body. Under normal physiological conditions, these nucleotides affected many biological functions of the liver inclusive of gluconeogenesis and lipid metabolism. Whereas in different disease settings, ATP and certain nucleotides acted as danger signals leading toward enhanced purinergic signaling in myriad of pathological processes (Vaughn *et al*., 2012).

2.6 Receptors for Purines and Pyrimidines in the Liver

It was shown that extracellular ATP and ADP caused release and uptake of Ca^{2+} into rat liver cells. Single cell measurements revealed intracellular release of Ca^{2+} in cell lines for rat liver epithelial cells stimulated by ATP. Purine nucleotides activated downstream Ca^{2+} signaling increasing the levels of InsP3 that might give clue of P2Y receptor involvement. On the other hand, ATP was found to stimulate the transepithelial secretion of potassium. To know the fact that this mechanism was operated through P2Y-Insp3 coupled pathway, suramin and 2-aminoethoxydiphenyl borate (InsP3 receptor inhibitor) was used to inhibit this effect. ADP does not affect $[Ca^{2+}]_i$ but increase cAMP giving a clue that P1 receptor were also involved (Burnstock *et al*., 2014)**.** There has been evidence that P2X4 receptors play important role in mediating ATP controlled transport of Na+ and Ca^{2+}.

2.7 Liver Cancer and Purinergic Signaling

Primary liver malignant tumors were almost always carcinomas and were further subdivided in hepatocarcinoma, bile duct carcinoma (cholangiocarcinoma) and hepatocholangiocarcinoma (Burnstock & Di Virgilio, 2013). Liver pathology was associated with chronic inflammation, altered metabolism, immune dysfunction and aberrant cell proliferation. It was observed that ATP infusions in intraperitoneal spaces of rat model of hepatocarinogenesis headed towards paraneoplastic foci in the liver. There was release of ATP from necrotic cells stimulates neighboring cells to die.

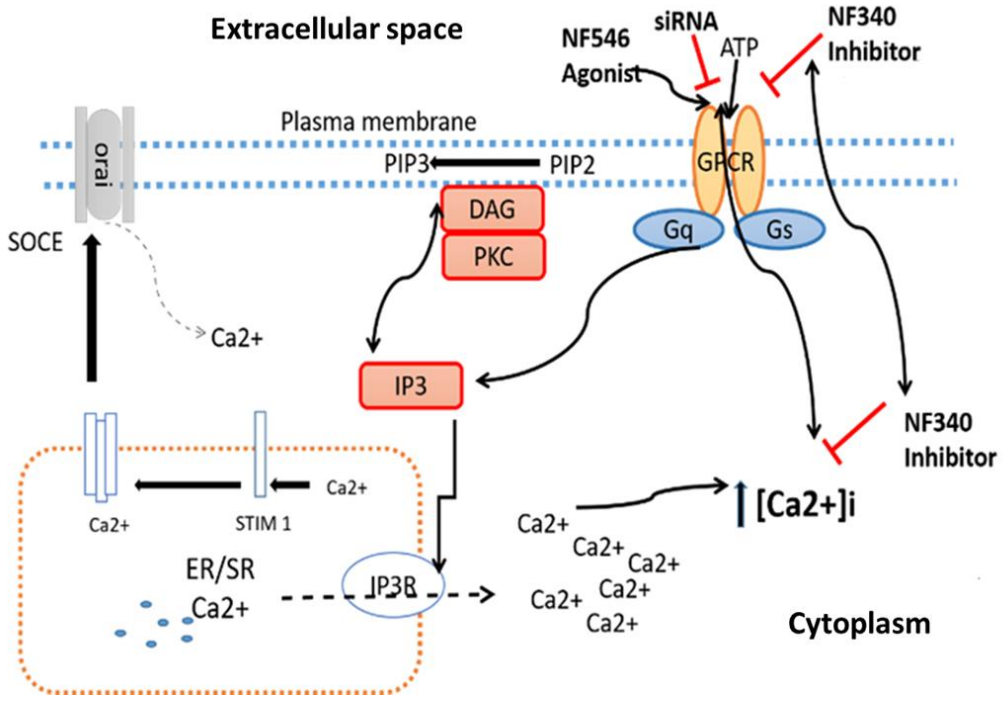


Figure 2.1 GPCR induced calcium signaling in Liver Cancer

Right, ATP binds GPCR and activates downstream IP3 that binds to the IP3R in the endoplasmic reticulum and causes intracellular release of calcium. Left, GPCR signaling in the presence of inhibitors.

2.8 P2X Receptor Signaling

2.8.1. Identification of P2X receptors

Burn stock, 1972, was the first to discover the concept of purinergic signaling with the idea of ATP acting as ligand to purino receptors. To date, P2X receptor family consists of seven subtypes P2RX1-7 that are found in different mammals and rodent species. All P2X receptors have been cloned in mid 90s and all subunits are 370 and 595 amino acids longs with sequence identity of more than 50%. They all have a common topology having two transmembrane (TM) domains with an intracellular N and C termini and a large extracellular ligand binding loop. The most important feature is the ten Cys residues that are bound with five disulfide bridges and found to be conserved in all vertebrate (Kaczmarek-Hájek *et al*., 2012). Different heterologous expression systems like Xenopus oocytes and mammalian HEK293 have been used that support the idea of hetro- and homomeric channel formation in P2X receptors (Fausther *et al*., 2012).

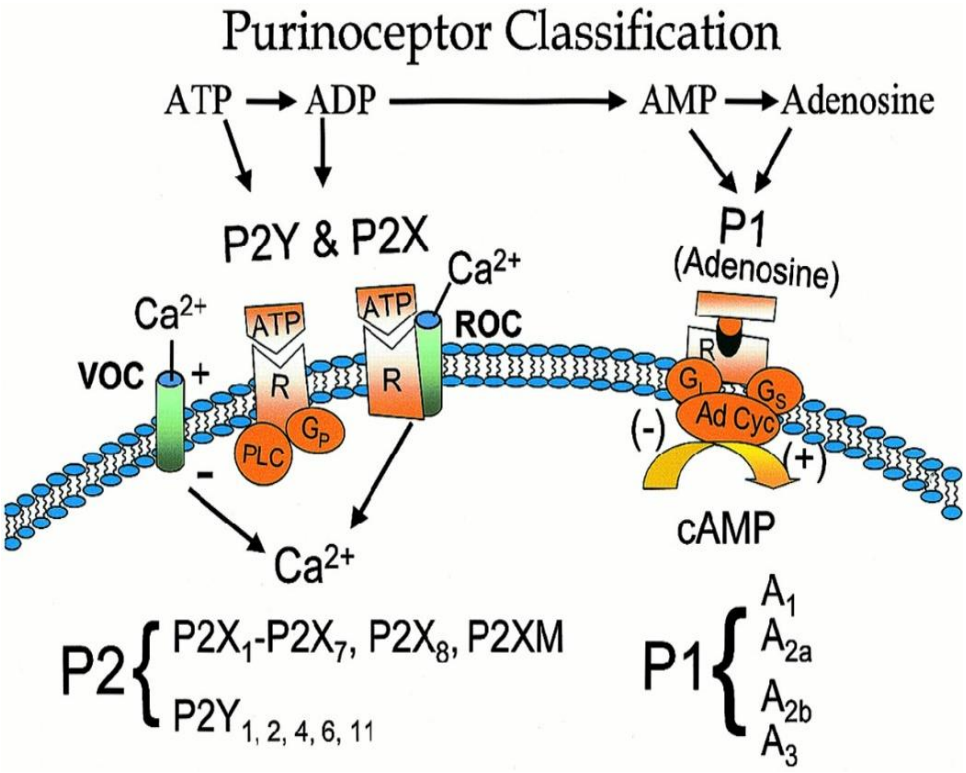


Fig 2.2 P1 and P2 receptor classification and the signaling mechanisms

PLC, phospholipase C; G_P, G proteins; R, receptor; VOC, voltage operated calcium channel; ROC, receptor-operated calcium channel; G_I, inhibitory G proteins; G_S, stimulatory G proteins; Ad Cyc, adenylate cyclase. Adapted from (Inscho, 2001).

Receptor	Agonists	Agonist binding affinities EC_{50}, μM	Main downstream signaling events	Expression
P2X receptors				
P2X1	ATP	0.05-1	Ca^{2+} and Na^{+} influx	Smooth muscle cells, megakaryocytes, platelets, lymphocytes, DCs, epithelial cells, ventricular myocardium, and neurons
P2X2	ATP	1-30	Ca^{2+} influx	Pancreatic cells, and neurons
P2X3	ATP	0.3-1	Cation influx	Neurons, keratinocytes, and $CD34^{+}$ hematopoietic precursors
P2X4	ATP	1-10	Ca^{2+} influx	Neurons, $CD34^{+}$ stem cells, lymphocytes, macrophages, MoDCs, fibroblasts, keratinocytes, and placenta
P2X5	ATP	1-10	Ion influx	Neurons, keratinocytes, and thyrocytes
P2X6	ATP	1-12	Ion influx	Neurons, keratinocytes, and thyrocytes
P2X7	ATP	> 100	Ca^{2+} and Na^{+} influx, K^{+} efflux pore formation	Neurons, lymphocytes, NK cells, neutrophils, monocytes, macrophages, DCs, and placenta
P2Y receptors				
P2Y1	ADP	8	PLC-β activation	Neurons, heart, skeletal muscle, platelets, liver, and digestive tract
P2Y2	ATP, UTP	0.1 (ATP), 0.2 (UTP)	PLC-β activation cAMP inhibition	Skeletal muscle, heart, lung, spleen, placenta, kidney, and neutrophils
P2Y4	UTP (ATP, UTP)	2.5	PLC-β activation cAMP inhibition	Intestine, lung, and placenta
P2Y6	UDP, UTP	0.3 (UDP), 6 (UTP)	PLC-β activation	Spleen, thymus, placenta, intestine, lung, and brain
P2Y11	ATP	17	cAMP production, PLC-β activation	Corneal epithelia, endothelial cells, pancreatic duct cells, DCs, and lymphocytes
P2Y12	ADP	0.07	cAMP inhibition	$CD34^{+}$ stem cells, mast cells, vascular smooth muscle cells, and platelets
P2Y13	ADP, ATP	0.06 (ADP), 0.26 (ATP)	cAMP inhibition	Bone marrow, spleen, liver, brain, airway epithelial cells, red blood cells, monocytes, DCs, and T lymphocytes
P2Y14	UDP-glucose	0.1-0.5	PLC-β activation	Hematopoietic cells, MoDCs, and human airway epithelial cells

Table 2.1 Expression patterns of P2X and P2Y receptor subtypes and their affinity for ATP. Adapted from (Vitiello *et al*., 2012).

2.8.2 ATP in physiological systems

ATP was found to be an extracellular messenger in very small volumes in resting cells or healthy tissues. ATP was present in intracellular compartment to very high quantities (from 5 to 10 mmol/l). It was degraded ubiquitous extracellular nucleotidases which allowed the swift generation of a perfect extracellular messenger molecule (Di Virgilio & Adinolfi, 2016).

2.8.3 Cellular targets of ATP and adenosine

The P2R family was classified into eight subunits of P2Y family from $P2Y_1$, $P2Y_2$, $P2Y_2$, and $P2Y_6$ to $P2Y_{11}$-$P2Y_{14}$. P2X comprised of seven subunits P2X1-P2X7. P2YRs were G-protein coupled receptors that mobilize Ca^{2+} and cyclic AMP generation/inhibition, as well as to stimulation of the ERK/MAPK pathway. P2XRs were homo/heterotrimeric ion channels that mediated transport of mono- (Na^+ and K^+) and divalent (Ca^{2+}) ions across the membrane (Di Virgilio & Adinolfi, 2016).

2.8.4 P2X receptors activation

Different pharmacological properties are used to categorize P2X receptors. Initially, different P2X receptors have variable potent dose of that range from nano molars (P2X1) to milli molars (P2X7). Second, ATP exposure for longer time led to desensitization of P2X receptors that fluctuated from milliseconds (P2X1) to more than twenty seconds for (P2X2-4 and P2X7) (Jarvis &Khakh, 2009). ATP analogues that acted as agonists (ex: α,β-MeATP; ATPγS) or antagonists (ex: TNP-ATP; PPDADS) were used to further characterize the P2X receptors using their pharmacokinetic properties. (Bianchi *et al*., 1999; Gever *et al*., 2006).

2.9 Pharmacology of P2X Receptors

2.9.1 P2X1 Receptors

These receptors were extensively expressed in platelets, urinary bladder, smooth muscles. It was found to mediate Ca^{2+} influx and appeared to involve in platelet shape change and aggregation. (Ford *et al*., 2006; Kennedy *et al*., 2007). 2-meSATP = ATP > *α*, *β*-meATP were the most important agonist at P2X1 receptors (Weber *et al.*, 2010). Suramin, a large polysulfonated compound, was antagonist at P2X1 receptor (Lalo *et al*., 2008). PPADS (Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid tetrasodium) was another antagonist at P2X1 receptor.

2.9.2 P2X2, P2X3 Receptors

BzATP, ATP, 2-meSATP were agonist for P2X receptors and P2X3. These are antagonized by suramin, PPADS (Burnstock, 2006a, 2006b; Burnstock & Kennedy, 2011; Burnstock & Knight, 2004).

2.10 P2X4 Receptors

This is widely expressed in various tissues including central and peripheral neurons, glandular tissues but with unclear functions. But pharmacological properties of P2X receptor vary depending on the specie. At present, the most encouraging therapeutic target is seems to be P2X4 in microglial cells due to its critical role in chronic neuropathic pain (Tsuda *et al*., 2003). It has been studied that P2X4 expression is increased in spinal nerves injury and inhibiting these receptors with antisense oligonucleotide greatly reduced the associated symptoms.

P2X receptor is stimulated by BzATP, ATP, and 2-meSATP. Ivermectin has been shown to potentiate the currents evoked by ATP, a distinguishing feature of P2X4 receptor as compared to other P2X receptors. Cibacron blue (Miller *et al*., 1998) and reactive blue 2.15 have also been shown to potentiate the sensitivity of the receptor.

P2X4 receptor agonist act in specie-dependent manner, as proven in various studies that the rat P2X4 receptor did not give results on antagonist specific for P2X receptors such as suramin and PPADS (Gever, *et al*., 2006; Jarvis & Khakh, 2009). 5-BDBD, a benzofurodiazepine derivative was found to antagonize P2X4 receptors at submicromolar concentrations (Nagata *et al*., 2009).

2.11 P2X5 AND P2X6 RECEPTORS

Various tissues express P2X5 receptors including central neurons, the eye, and cardiac tissues. P2X5 receptor is activated by ATP and 2-meSATP. Human isoforms own BzATP, α, β-meATP as agonist (Wildman *et al*., 2002). This agoist action is blocked nonselective antagonists PPADS >suramin in both rat and human (Khakh *et al*., 2001). For P2X6 receptor, ATP and α, β-meATP acted as full agonist while PPADS were antagonists (Lê *et al*., 1998).

2.12 P2X7 RECEPTORS

This receptor is widely expressed by brain cells and immune cells including macrophages, white blood cells, mast cells etc (Burnstock, 2007; Burnstock & Kennedy, 2011; Burnstock & Knight, 2004). P2X7 receptor, a ligand-gated cation channel, get sensitized with agonist and rapid inward currents leads to change in intracellular enzyme activity. This enzyme activity leads to activation of G-protein coupled receptors and downstream signaling molecules noticeably proinflammatory cytokines, interleukin-1β and interleukin-18. P2X7 made an important block with NLRP3 inflammasome complex, a protein complex that initiates the production and release of interleukin-1β (Ghiringhelli *et al*., 2009). For the reason, it is thought to be a potential therapeutic target in a number of diseases such as chronic inflammatory disorders, rheumatoid arthritis and osteoarthritis.

P2X7 receptor activation mainly depends on higher magnitudes of ATP in milli molar quantity than for the other P2X subtypes. BzATP acted as a potent agonist at P2X7 and showed increased potency when divalent cations are removed. (Jarvis & Khakh, 2009).PPADS were found to antagonizes human, rat, and mouse P2X7 receptors. On the other hand, suramin was found to have flexible or no effective inhibition in any species (Gever *et al*., 2006; Jarvis & Khakh, 2009).

2.13 P2Y receptor Signaling

On the basis of sequence similarity G-coupled proteins, the hetromeric group of protein, were broadly divided into four subfamilies (G_q, G_s, $G_{i/o}$, and $G_{12/13}$) (Neves *et al*., 2002). P2Y receptor family members include $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ that are G_q-coupled, show 28–52% sequence homology while $P2Y_{12}$ $P2Y_{13}$, and $P2Y_{14}$ receptor family members that are $G_{i/o}$-coupled show 45–50% sequence homology.

Group	Receptor	Chromosome (human)	Agonist (human)	G protein
A	$P2Y_1$	3q24-25	ADP	G_q
	$P2Y_2$	11q13.5	ATP = UTP	G_q (+G_i)
	$P2Y_4$	Xq13	UTP	G_q (+G_i)
	$P2Y_6$	11q13.5	UDP	G_q
	$P2Y_{11}$	19p31	ATP	$G_q + G_s$
B	$P2Y_{12}$	3q21-25	ADP	G_i
	$P2Y_{13}$	3q24-25	ADP	G_i
	$P2Y_{14}$	3q24-25	UDP-glucose	G_i
			UDP	

Table 2.2 properties of P2Y receptors Adapted from (Boeynaems *et al*., 2012).

2.14 Activation mechanisms P2Y family subunits

2.14.1 $P2Y_1$ and $P2Y_{12}$ receptors

As $P2Y_1$ coupled to G_q was found to activate the phospholipase C β and mobilize intracellular Ca^{2+} (Soulet *et al*., 2005). While $P2Y_{12}$ coupled to G_{i2} found to inhibit adenylyl cyclase and activation of PI3K, Akt, Rap1b, and potassium channels. When $P2Y_1$ and $P2Y_{12}$ receptors together were activated by ADP, they were found to control platelet aggregation and their shape change (Dorsam & Kunapuli, 2004).

2.14.2 $P2Y_2$ Receptors

$P2Y_2$ receptor was found to be activated in the same way by ATP or UTP while coupling to G_q, G_o, and G_{12} proteins. (Bagchi *et al*., 2005; Liao *et al*., 2007). Activation of G_o by the $P2Y_2$ receptor led to activation of RhoA, whereas activation of G_{12} led to activation Rac (Murthy & Makhlouf, 1998).

2.14.3 $P2Y_{11}$ Receptors

$P2Y_{11}$ receptor was activated by ATP/UTP coupled to members of the G_q and G_s. G_s proteins stimulate adenylyl cyclase to increase production of cAMP. $P2Y_{11}$ receptor coupling to G_s is important for controlling several physiological processes (Kaufmann *et al*., 2005; Nguyen *et al*., 2001; Vaughan *et al*., 2007).

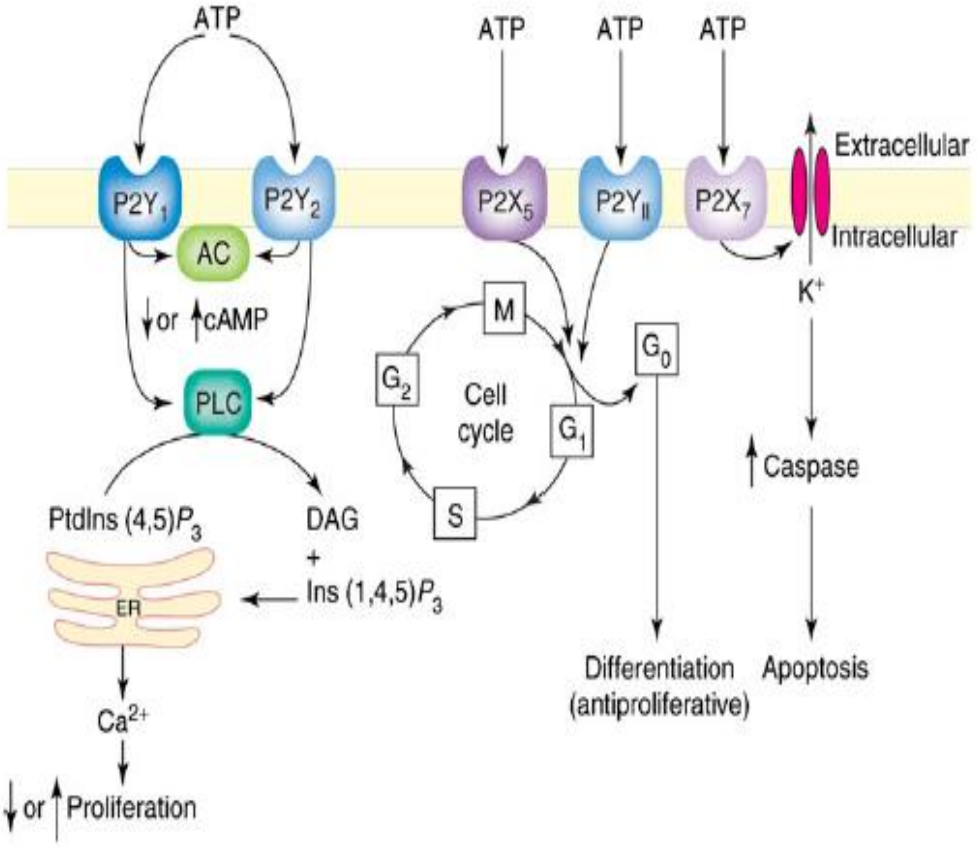


Fig. 2.3 Schematic diagram representing P2 receptor mechanisms in cancers.

P2Y1 and P2Y2 receptors effect cell proliferation through PLC pathway. P2X5 and P2Y11 receptors switch cell proliferation into differentiation state. P2X7 activates apoptotic pathway. Adapted from (Geoffrey Burnstock, 2006b).

Chapter 3

MATERIALS AND METHODS

3.1 Collection of data and samples

Ex-planted liver tissue samples of patients suffering from Chronic Hepatitis, HCC and cirrhosis along with the patient history, age, sex, ALP/ALT, AFP levels and tissue status (cirrhotic, tumor or hepatitis) was obtained from Hepatobiliary/Liver Transplant Unit Shaikh Zayed Hospital, Lahore. Patients from all over Punjab, Lahore. They provided us with the formalin fixed paraffin embedded (FFPE) liver biopsy & liver tissue sections. The tissue sections comprising of 3 μm in thickness, were heat-fixed upon the immunohistochemistry glass slides at the Histopathology Lab, Sheikh Zayed Hospital Lahore. Samples were transported in glass slide storage box and stored at room temperature.

3.2 Immunohistochemistry

For the evaluation of purinergic receptors the tissue sections were taken from Histopathology Lab, Sheikh Zayed Hospital Lahore. The tissue sections are fixed upon glass slides for Immunohistochemistry (IHC).

3.2.1 Fixation

The tissue biopsies were cut into 4 μm thickness by using microtome. Poly- L lysine glass slides were used to fix the tissue through heat fixation and further incubated for one hour at 60ºC.

3.2.2 Deparaffinization of samples

All the biopsy samples were deparaffinized. For this purpose, 100 % Xylene solution was used for 20 minutes to remove any residual paraffin from the samples. To further rehydrate the sample tissues, ethanol solution was used from higher 100% to lower

concentration 30%. To avoid any drying and non-specific background staining, slides were kept in running water.

3.2.3 Epitope Retrieval

There can be loss of immunoreactivity of epitopes during fixing process. Epitopes become unreactive owing to protein crosslinking and masked antigenic sites. To avoid any masking, slides were kept in antigenic retrieval solution that consist of sodium citrate buffer and Tween 20. Samples were kept in microwave oven for 10 mins and then cooled for 35 min at room temperature. 1X P.B.S was used to wash the slides.

3.2.4 Blocking

Hydrogen peroxide (H_2O_2) was used to prevent the binding of nonspecific binding of antibodies. The slides were placed in a humidity chamber to keep them in moist environment. And latter, blocking solution was removed by rinsing with simple water.

3.2.5 Primary and secondary Antibody Incubation

Anti-P2X4 (ab134559, abcam UK) and anti-P2X7 (ab93354, abcam UK) was used after dilution in 5% BSA. This was then applied on the tissue samples and kept at room temperature for 2 hrs. These samples were washed to remove any residual primary antibody for further incubation with HRP labelled secondary antibody (ab97110, abcam UK) and kept in dark tray for 1 hour at room temperature.

3.2.6 Detection

HRP Label, DAB substrate was used to visualize the binding of secondary antibody with the primary antibody and detection was based on color change. The kit consist of DAB substrate and DAB chromogen (Abcam Cat: ab64238, UK) that was applied to tissue samples followed by incubation of 5-10mintues at room temperature and stain was washed using tap water. To stain the nuclei of the cells specifically, hemotoxylin Stain was used. To visualize the slides, Labomed TCM400 inverted microscope (Labo America Inc., USA) was used.

3.2.7 Evaluation of immuno histochemical findings

A pathologist with no knowledge of clinical and outcome data was called to analyze the slides. Brown color was defined as positive staining as observed in hepatocytes All positive cells were measured under different magnifications of 10X, 20X, 40X and 100X.

3.3 Modelling of human P2X4 using zebrafish P2X4 model as template

Molecular operating environment (MOE) software was used to align the sequence of P2X4R obtained from uniport. And P2X4 zebrafish structure (ID: 3I5D) was used as template to model human P2X4R (Nicke, 2012) from MODELLER (Eswar, 2006). There were fifty models that were clustered into groups. These groups were made on the basis of their RMSD values. ERRAT scores, MODELLER and their potential energies were used to finalize the representative models.

3.3.1 Protein-Protein interaction

PPI of P2X4 and E1 was done using docking. Docking software was used for this purpose named as, High ambiguity driven biomolecular docking (HADDOCK) (Vriesetal, 2010). All the possible interactive residues were calculated in the CPORT.

3.4 Cell culture

Huh-7 cells were maintained in the specific media specific for Huh-7 cells. DMEM supplemented with penicillin and streptomycin and 15% heat inactivated fetal bovine serum was used. HEK 293 cells were kept in complete medium supplied with 200ng/ml G418. Cells were maintained at 37°C temperature in a cell culture incubator supplied with 5% CO_2.

3.5 Intracellular Ca^{2+} measurement

Standard buffer solution (SBS) is used that contains in grams (g) 7.55NaCl, 0.37 KCl, 1.44 Glucose, 2.38 HEPES, 1.2MgCl2, 1.5ml CaCl2.

Mix Fura-2 AM 50 µg lyophilized by adding 50 µL of DMSO and vortex for 1 min. Store at -20 ºC wrapped in foil for light protection. 10 µl of 1 mM Fura-2 was mixed with 10 µlPluronic F-127. Cells grown in 96-well plates were washed with SBS at 80 µl/well 2 times.Fura-2 AM was added in the loading solution at 50 µl/well using a multichannel pipette and incubated the plate at 37 °C for 1 hr. Cells were washed 3 times with the SBS. After the last wash, leave 80 µl of the SBS buffer in each well. When the cells are in loading period, we prepared a series of 3x dilutions of agonists (or antagonists) in the SBS assay buffer. Diluted compound was added in 250- to a 96-well compound plate. Flex station III software was used to save the experiment on the computer system.

3.6 Immunostaining

Cells were harvested as described in section 2.2.1 and were plated into a 24-well microplate at 5 x 10^3 cells per well. After 24 hours incubation, cells were treated with different concentrations of compounds indicated in the Results section and stained as follows. After treatment for 2 hours or 24 hours, cells were incubated in 200µl per well pre-warmed 2% PFA for 15 for minutes. After giving wash with PBS thrice, cells were kept in 200µl solution used for blocking for 15 min, and then with Alexa Fluor®488 Phalloidin solution (diluted at 1:1000 in blocking solution) for one hour at room temperature or overnight at 4ºC in dark. Cells were washed extensively with 0.5% Tween-20 washing solution in dark three times and with PBS. A droplet of mounting media with DAPI was placed on a glass slide in dark. Cells were washed

with distilled water in dark to remove PBS. Coverslips were removed from the well, mounted with the cells facing the mounting media in the slide, and the slides were stored in 4^{o}C overnight. Fluorescent images were captured using confocal microscope LSM 700 (Carl Zeiss) using ZEN software, or using EVOS® Cell Imaging System (Thermo Fisher Scientific). For each condition, three wells of cells and three regions for each well were taken using confocal microscope or nine regions for each well using EVOS® Cell Imaging System. The same image excited at358 nm (ultraviolet) and 496 nm respectively, andwere captured separately by transmitted light channel, blue (390-400 nm) and red fluorescent channels (586-615nm), respectively. Images were merged by Image J.

3.7 Cell migration assay

Cell migration assay was performed in plates with 24-wells. These plates have special wells that have pores of 8um (BD Biosciences). Huh-7 cells ($1 \times 10^{5)}$ were seeded in the upper chamber. Both compartments were supplied with media containing 10%, which represent the control conditions. Agonist and antagonists were added in the lower and upper chamber respectively. Cells were incubation at 37°C and 5% CO_2 for 24 hr, and fixed with 4% paraformaldehyde and stained with 0.05% crystal violet for 30 min at room temperature. Nine different areas were selected and images were taken on ECLIPSE TE2000-U (Nikon) then counted using Image J software. Cell migration was presented by expressing the migrated cell number as % of that under control conditions for significant comparisons between different experiments.

3.8 Cell death assay

Cell viability assay was performed by using ATP. Cells were seeded on 24- well plated at 1x 10^4 cells per well and incubated in cultured medium. Dulbecco Modified

Eagle's Medium (DMEM) was used with the addition of penicillin and streptomycin without or with supplementation of ATP for 24 hr. Cells were stained using 5 μg/ml propidium iodide (PI) and 1 μg/ml Hoechst for 30 min. Images were captured with EVOS Cell Imaging System (Thermo Fisher Scientific).

3.9 Cell Proliferation Assays

To examine the effects of ATP and inhibitors on the cell viability, these cells were seeded on 24-well plated at 1×10^4 cells per well and incubated in culture medium for 0, 24, 48 or 72 hr. Cell proliferation is quantified by counting % confluency of cell images over the time using by using IncuCyte ZOOM® live-cell imaging.

3.10 BRN Modeling By Using GENOTECH

Biological complex systems are addressed by a number of traditional approaches. Current study have been working on same grounds as (Khalid *et al*., 2016). All biological observations were used to construct discrete models and these observations are then applied through the software, GENOTECH. Nodes and edges are main components of biological regulatory network. Directed arrows are used to connect all the nodes that represent activated and inhibitory actions. Any genetic changes in the signaling network are determined through dynamic networking fall under BRN.

3.11 Simulation by using Jimena

Java genetic regulatory network, Jimena is used for simulation networks by using new algorithms and models surrounding GRNs. First, a yED graphml file is created containing network. All the activations are modeled using the yED standard arrow tip, inhibiting influences using any other tip (e.g. a diamond). Parameters of the interactions such as the weight of an input, SQUAD's sigmoid gain etc. can be set after the network was loaded into Jimena

3.12 Data Presentation and Statistical Analysis.

Statistical analysis was performed using Student's *t*-test for two groups. One-way ANOVA was used for comparing two groups using Origin software. All data was represented as standard error mean with $p < 0.05$ was considered as significant.

Chapter 4

RESULTS

4.1 Expression Profile of Purinergic Receptors P2X4 and P2X7

Fifty (50) paraffin embedded samples from explanted liver tissue samples of HCC were collected. P2X4 and P2X7 expression was observed through immuno-histochemical analysis. We measured the expression of P2X4 and P2X7 receptors in HCV HCC and non-HCV HCC patient's samples (Fig 4.1). We categorized samples into 1) HCV HCC includes twenty eight (28) patient's samples of ex-planted liver tissues 2) non-HCV HCC includes twenty two (22) patient's samples of ex-planted liver tissues.

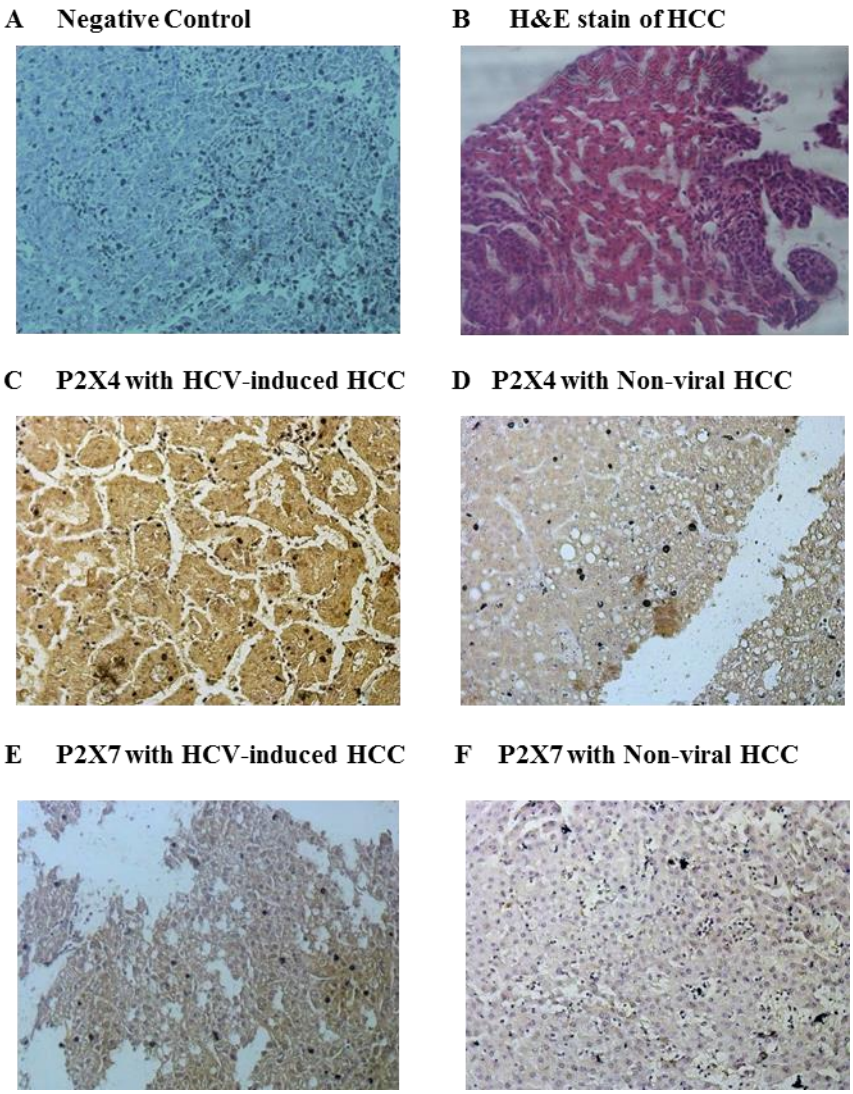


Fig. 4.1 Immuno-histochemical staining of explanted liver tissue samples (HCC and HCV induced HCC with P2X4 and P2X7 antibody.

A) Negative control stained without primary antibody B) H& E stain of HCC tissue to show liver architecture. C) P2X4 expression in HCV induced HCC tissue samples. D) P2X4 expression in explanted liver HCC tissue E) Expression of P2X7 in HCV induced HCC tissue samples. F) P2X7 expression in explanted liver HCC tissues. Brown color shows positive expression and blue color shows no expression. All micrographs were captured at 200X magnification.

Among 28 patients of HCV induced HCC, 5/28 (17.8%) showed low expression of P2X4 and 23/28 (82.1%) showed moderate expression of P2X4 in HCV HCC. Among 22 patients in non-HCV HCC, 15/22 (68%) showed low expression of P2X4 and 7/22 (31%) showed moderate expression of P2X4 in non-HCV HCC (Fig. 4.2a). There was a significant increase in the P2X4 receptor expression in HCV HCC as compared to non-HCV HCC. This moderate expression of P2X4 can be correlated with cellular insult owing to virus that may cause superfluous release of ATP in the cellular environment. Although liver cells express P2X4 and P2X7 at basal levels (Aurore Besnard *et al*., 2016) but their levels vary in disease condition depending on the pathology of disease. The current study is correlated with our previous findings of Manzoor *et al* that shows increased levels of various variants of P2XR while in the presence of HCV envelop E1E2 proteins (Manzoor *et al*., 2011).

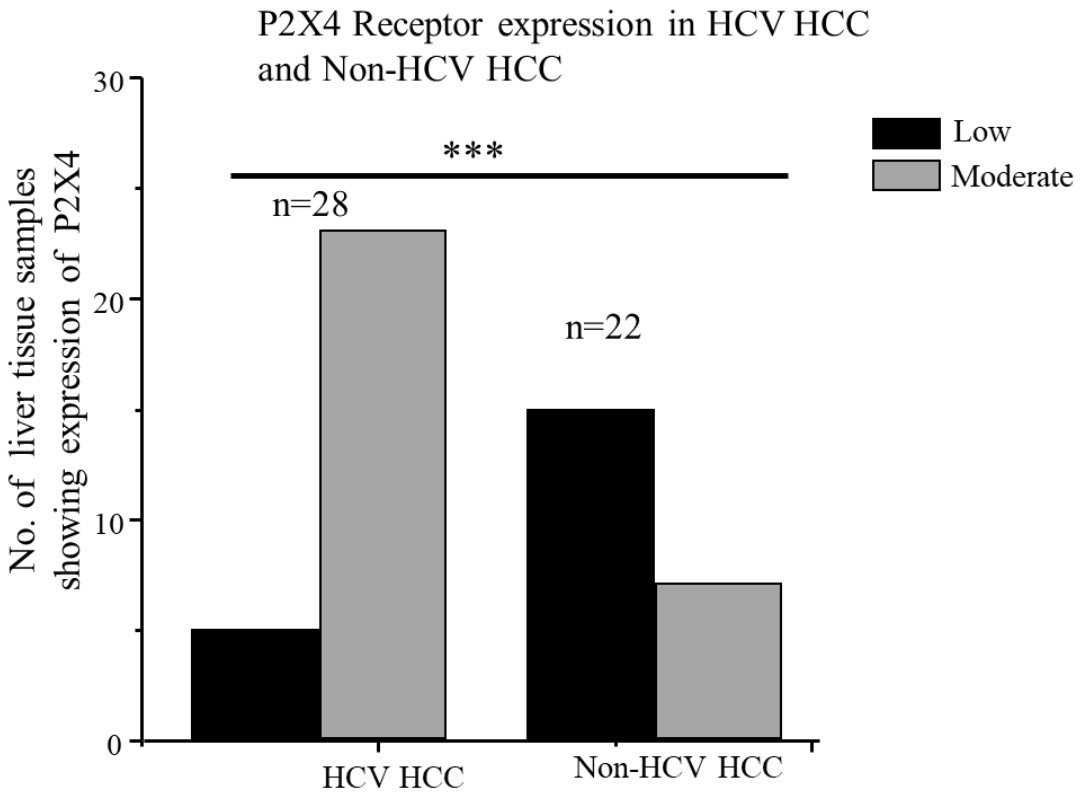


Fig 4.2a Expression of P2X4 receptor in HCV and non- HCV HCC.

This figure shows P2X4 expression from low (17%) to moderate (82%) in HCV HCC and P2X4 expression in non-HCV HCC from low (68%) to moderate (31%).

Similarly when we measured the expression of P2X7 in HCV HCC, among 28 patients in HCV HCC, 21/28 (75%) showed low expression of P2X7 and 7/28 (25%) showed moderate expression of P2X7. Whereas among 22 patients in non-HCV HCC, 14/22 (63%) showed low expression of P2X7 and 8/22 (36%) showed moderate expression of P2X7 in non-HCV HCC (4.2b). P2X7 receptor expression was almost un-responsive in both HVC HCC and non-HCV HCC. This might give us a clue that P2X7 is most widely expressed on peripheral blood mononuclear cells (PBMCs) and other immune cells that's why play a significant role in inflammatory response of immune cells. It has been studied previously that increased expression and activation of P2X7 was found to help to provoke the immune system to eradicate the viral infection from the body (Ashraf *et al*., 2013).

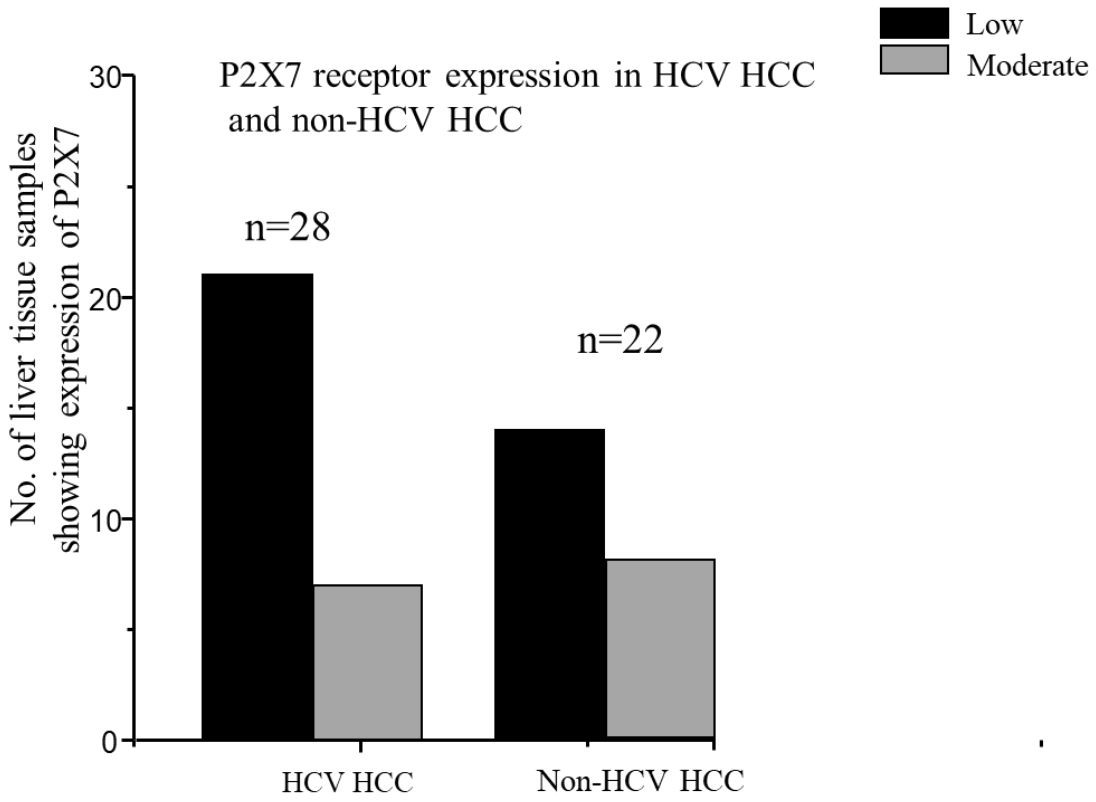


Fig 4.2b Expression of P2X7 receptor in HCV and non- HCV HCC.

This figure shows P2X7 expression from low (75%) to moderate (25%) in HCV HCC and P2X7 expression from low (63%) to moderate (36%) in non-HCV HCC.

4.2 *In Silico* Analysis of P2X4 Protein

In our first part of the study, P2X4 receptor was found to have pro-viral role. It has also been established previously that cell lines stably expressing HCV envelop protein E1E2 have improved P2X4 receptor expression. To further, assess whether this receptor has any role in viral entry, we devised PPI studies of P2X4 receptor with viral entry protein HCV E1 to lay down the basis for the viral exploitation of these receptors to augment the disease pathology.

4.3 Comparative Modeling of P2X4 Protein

To evaluate the interaction of envelop protein E1 of HCV with P2X4R, comparative modeling of P2X4 protein of zebrafish was performed. Crystal structure of P2X4 zebrafish (ID: 3I5D) was used as template to create homology models of human P2X4 receptor protein. Total of 50 models were generated by using MODELLER. On the basis of scores provided by ERRAT, QMEAN and MODELLER ten models were selected for further analysis (Table 4.1). ERRAT scores were in the range of ~70-80. Best model was selected on the basis of highest ERRATs score (58.592) and lower energy values.

Selected Models	**Errat score/overall quality factor**	**Selected Models**	**Errat score/overall quality factor**
model Q99571.B99990001	55.636	model Q99571.B99990004	58.282
model Q99571.B99990002	55.901	model Q99571.B99990005	56.269
model Q99571.B99990003	52.844	model Q99571.B99990006	58.592
model Q99571.B999900010	55.855	model Q99571.B99990007	54.037
model Q99571.B99990009	53.679	model Q99571.B99990008	53.998

Table 4.1 Selected homology models for human P2X4 receptor protein.

4.4 Protein–Protein Interaction Studies of HCV Envelop Protein E1 and P2X4

The structure was analyzed in pymol for tracing out the possible interactions in docked protein. PPI studies of P2X4 and E1 revealed that Viral E1 might competitively bind liver cell surface receptors through polar contacts between amino acid residues at a distance of 3.9 $A^{0.}$ We identified amino acid residues in the C-terminal of P2X4 receptor protein were Gly-960 , Gly-641, ASP-974, ASP-648 and amino acids in the N-terminal of viral E1 were ALA-17, ASN-12, MET-5, ARG-4, HIS-3 (Fig.4.3a)

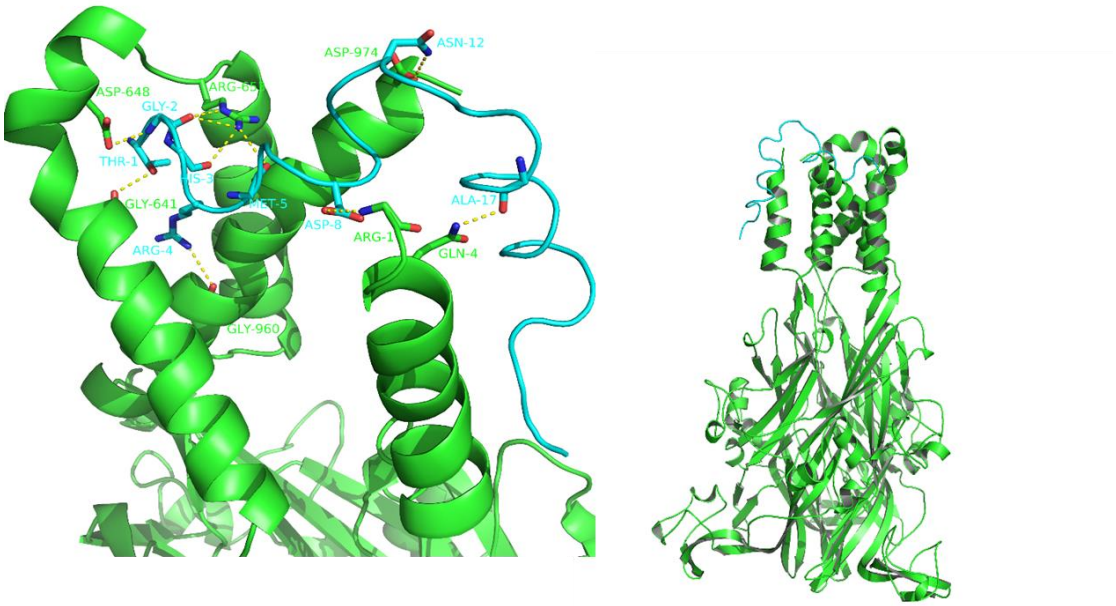


Fig 4.3a Crystal structure of the docked liver receptor P2X4 and Viral E1 protein.

Best structures are shown above with possible interactions between different polar sites. Overview of the complex showing interactions between P2X4 and E1 at a distance of 3.90 Angstrom distance. P2X4 is a large protein of approx. 970 residue long shown in green and E1 is a small protein of 26 residues shown in blue. Active residues involved in the interaction are highlighted.

Already available Crystal structure of P2X7 (Caseley *et al*., 2015) was auto docked with viral envelop E1 protein. We docked the protein at lowest energy and most populated area and amino acids were identified. Lower energy P2X7 receptor's interactions were ASN-187, Gly-185, ARG-244, PHE-240 with HCV E1 protein residues naming THR-16, ASP-8, ARG-26 respectively. And most populated interaction between P2X7 and E1 protein within 3.9 A^0 was TYR-317 and ASN-12 (Fig.4.3b).

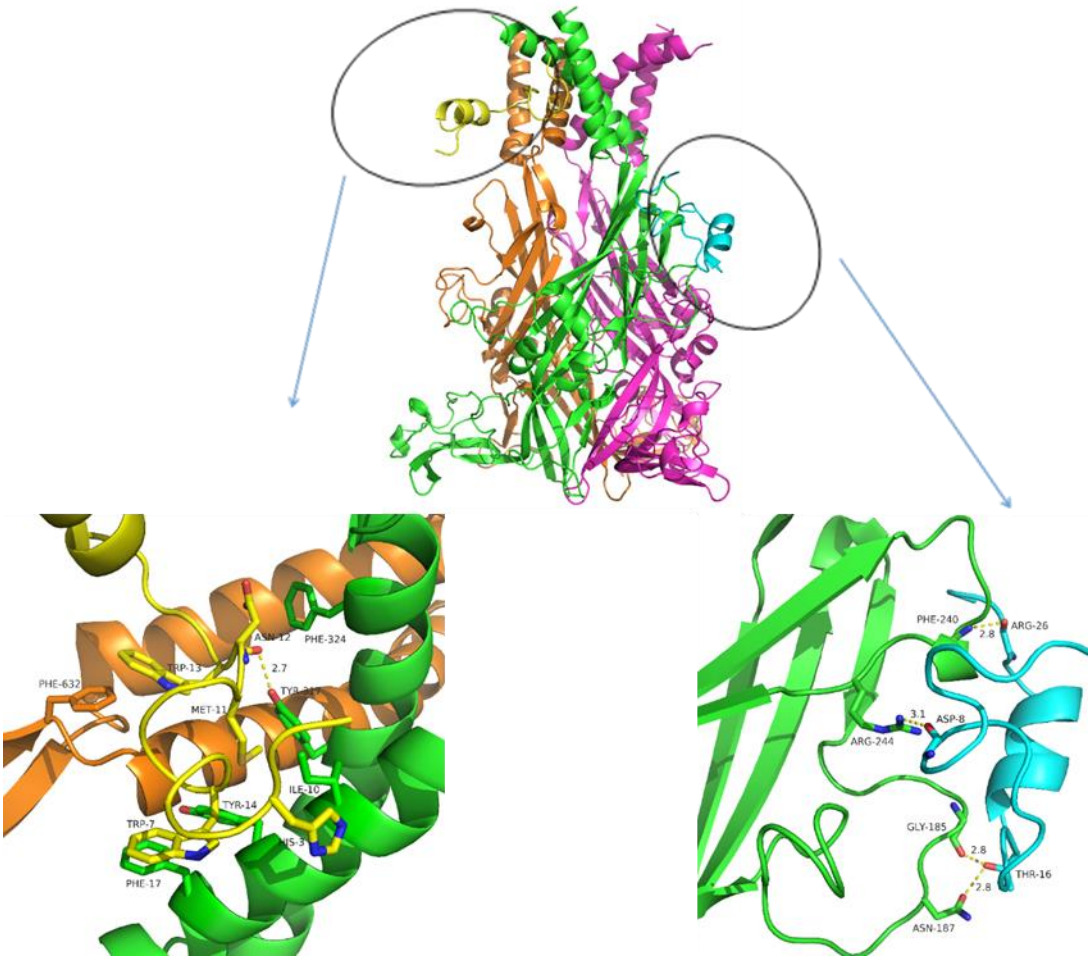


Fig 4.3b Crystal structure of the docked liver receptor P2X7 and Viral E1 protein.

Best structures are shown above with possible interactions between different polar sites. Overview of the complex showing interactions between P2X7 and E1 at a distance of 3.90 Angstrom distance. P2X7 is a large protein of approximately 970 residue long shown in green at lowest energy site and in brown color at most populated site and E1, a small protein of 26 residues, is blue at lowest energy site and yellow at most populated site. Active residues involved in the interactions are highlighted.

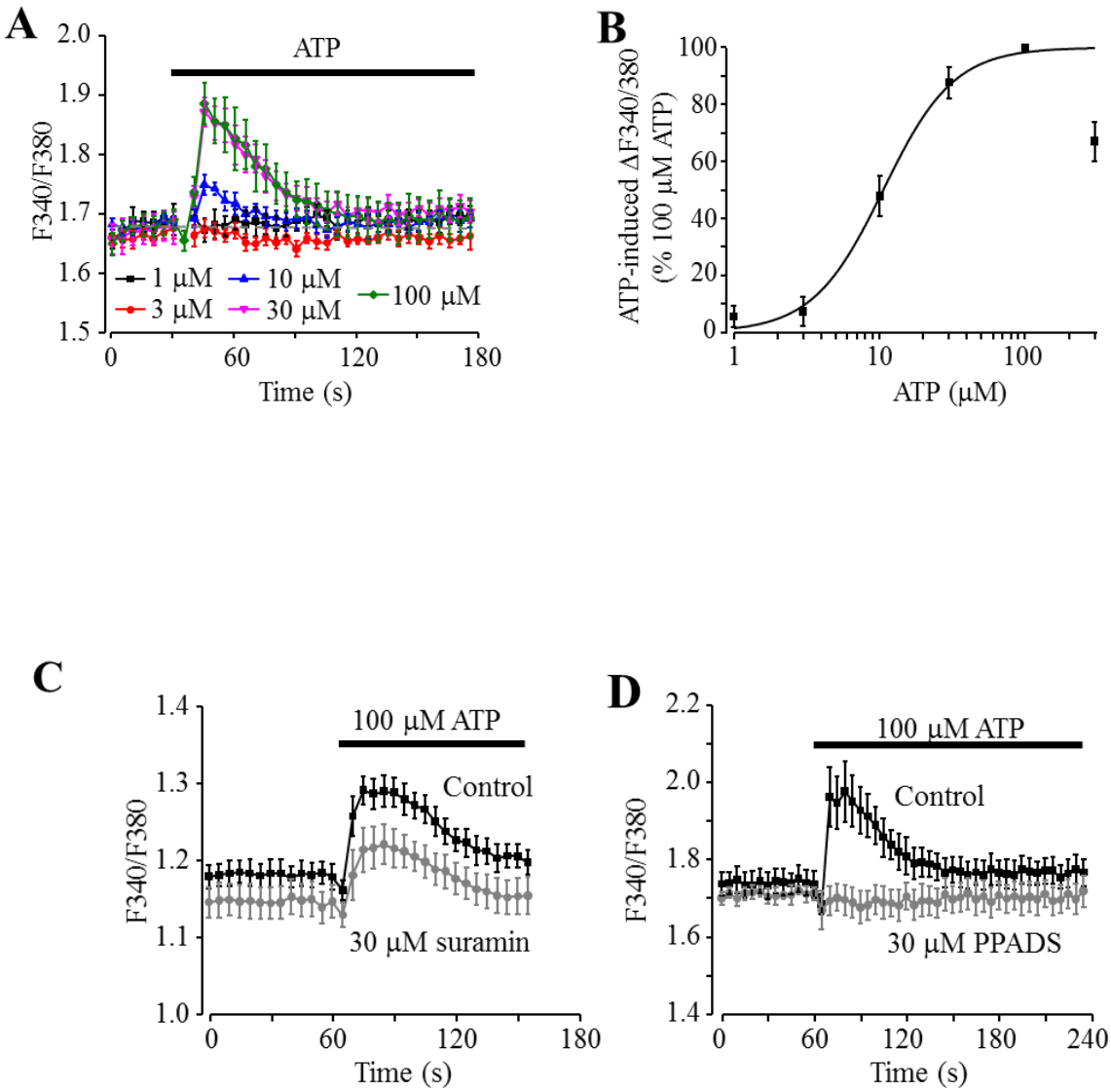
4.5 Determination of Functional activity of P2 Receptors

ATP acts as ligand for all P2X receptors are activated by ATP with different potency. This will allow to form an ion-conducting pathway across the plasma membrane that allows passage of cations including Ca^{2+}. P2Y receptors have G-protein coupled receptors that in turn activate phospholipase C (PLC) and IP3R in endoplasmic reticulum to trigger the release of Ca^{2+}. While P2X receptors stimulate extracellular calcium influx on binding with ligands. We will examine the P2 receptor (P2X and P2Y) for ATP stimulated Ca^{2+} signaling in human hepatocellular carcinoma cells. Huh-7 cells induced with different conc. of ATP will be used to measure any change in calcium. Agonist and antagonist specific for the receptors will be used to measure the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) using fura-2-based ratiometry. In addition, we will find out the strong increase or up-regulation of specific P2 receptor in human HCC tissues. And Carcinoma-specific increase in the expression of P2 receptor will be narrowed down and its role in liver cell migration will be observed.

4.6 ATP induces a robust increase in the $[Ca^{2+}]_i$ in Huh-7 cell

ATP-induced signaling in Huh-7 cell was done by measuring Ca^{2+}responses in the extracellular Ca^{2+}-containing solutions after treating Huh-7 cells with ATP using fura-2 based ratiometry and FLEX-station. ATP applied at 1-300 µM induced an increases in the $[Ca^{2+}]_i$ in a concentration-dependent manner (Fig 4.4A and 4.4B). The increase reached the maximum at 100 µM ATP, and the increase induced by 300 µM ATP were reduced, which may result from receptor desensitization. After data fitting to Hill equation produced an EC_{50} of 11 µM and Hill coefficient of 1.8 (Fig 4.4B). Pre-treatment with 30 and 100 µM suramin (Fig 4.4 C and E) or with 30 µM PPADS, two

P2 receptor genic antagonists, strongly inhibited or almost completely abolished ATP-induced Ca^{2+} responses (Fig 4.4D and 4.4F). As a consequence, we got first indication that ATP can increase in the $[Ca^{2+}]_i$ in Huh-7 cell via the P2 receptor. Activation of the P2X receptor ion channels can increase the $[Ca^{2+}]_i$ as a result of extracellular Ca^{2+} influx. Protein expression of the P2X7 receptor in Huh-7 cell was previously reported and confirmed using immunocytochemistry (Fig 4.8A).


A
ATP
F340/F380
1 µM
3 µM
10 µM
30 µM
100 µM
Time (s)
B
ATP-induced ΔF340/380
(% 100 µM ATP)
ATP (µM)
C
100 µM ATP
Control
30 µM suramin
F340/F380
Time (s)
D
100 µM ATP
Control
30 µM PPADS
F340/F380
Time (s)

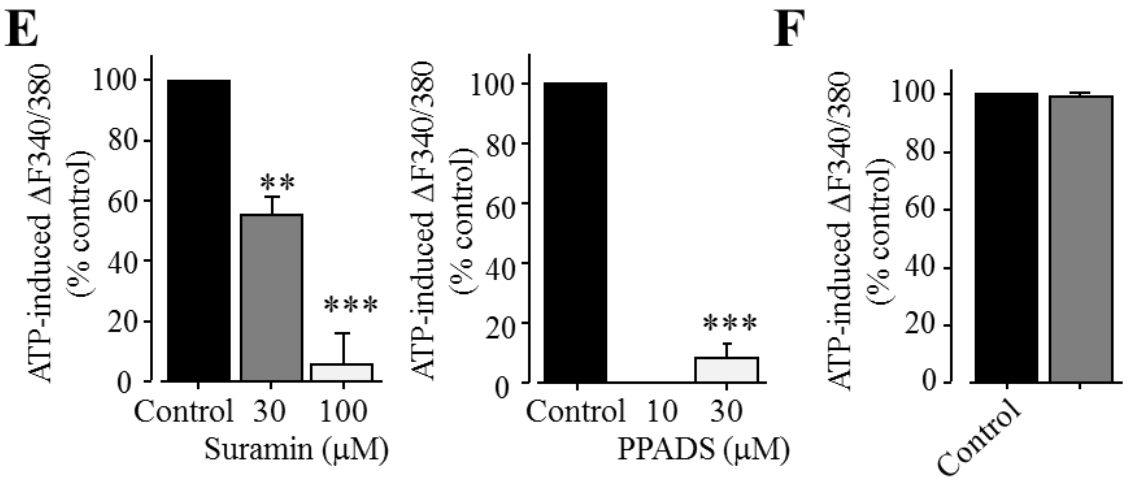


Fig 4.4 ATP induces robust increase in the $[Ca^{2+}]_i$ in Huh-7 cells**.**

Illustrative records of Ca^{2+} responses induced by 1-100 μM ATP. (B) ATP concentration-peak Ca^{2+} response curve, constructed by expressing ATP-induced responses as % of that induced by 100 μM ATP. The smooth line represents Hill equation with EC_{50} and Hill coefficient of 11 μM ATP and 1.8, respectively. (C) Representative records of Ca^{2+} response in the presence of 100 μM ATP, in control cells or cells pre-treated with 30 μM suramin. (D) Typical records of Ca^{2+} response by100 μM ATP, in control cells or cells with pre-treatment of 30 μM PPADS (E) Summary of ATP stimulated escalations in the $[Ca^{2+}]_i$ in control and cells treated with indicated concentrations of 30 and 100 μM suramin. (F) Summary of ATP-induced peak increases in the $[Ca^{2+}]_i$ in control and cells treated with 30 μM PPADS, expressed as % of that in control cells (F) Summary of ATP-induced peak increases in the $[Ca^{2+}]_i$ in control and cells with treatment of 10 μM 5-BDBD, expressed as % of that in control cells.

However, it is noticeable that application of 100 and 300 µM 2',3'-O-(4-benzoylbenzoyl)-ATP (BzATP), an agonist more potent than ATP at the human P2X7 receptor and also activating other P2X receptor , was found to fail in inducing any detectable Ca^{2+} response (Fig 4.5A) that suggests meager expression of the P2X7 receptor functionally. α-β methylene ATP potently activates the human P2X receptors containing P2X1, P2X3 or P2X5 subunit. There was no discernible Ca^{2+} response to 100 µM αβmeATP (Fig 4.5B). A previous study shows functional P2X4 receptor expression functionally in rodent HCC cells (Zimmermann H, 2000).10 µM 5-BDBD, a selective P2X4 receptor antagonist with a sub micro molar potency (Jiang, 2010), however, resulted in no inhibition of ATP-induced increase in the $[Ca^{2+}]_i$ (Fig 4.5C), suggesting no major role for the P2X4 receptor in Huh-7 cell. There were no positive results for immunocytochemistry for P2X4.

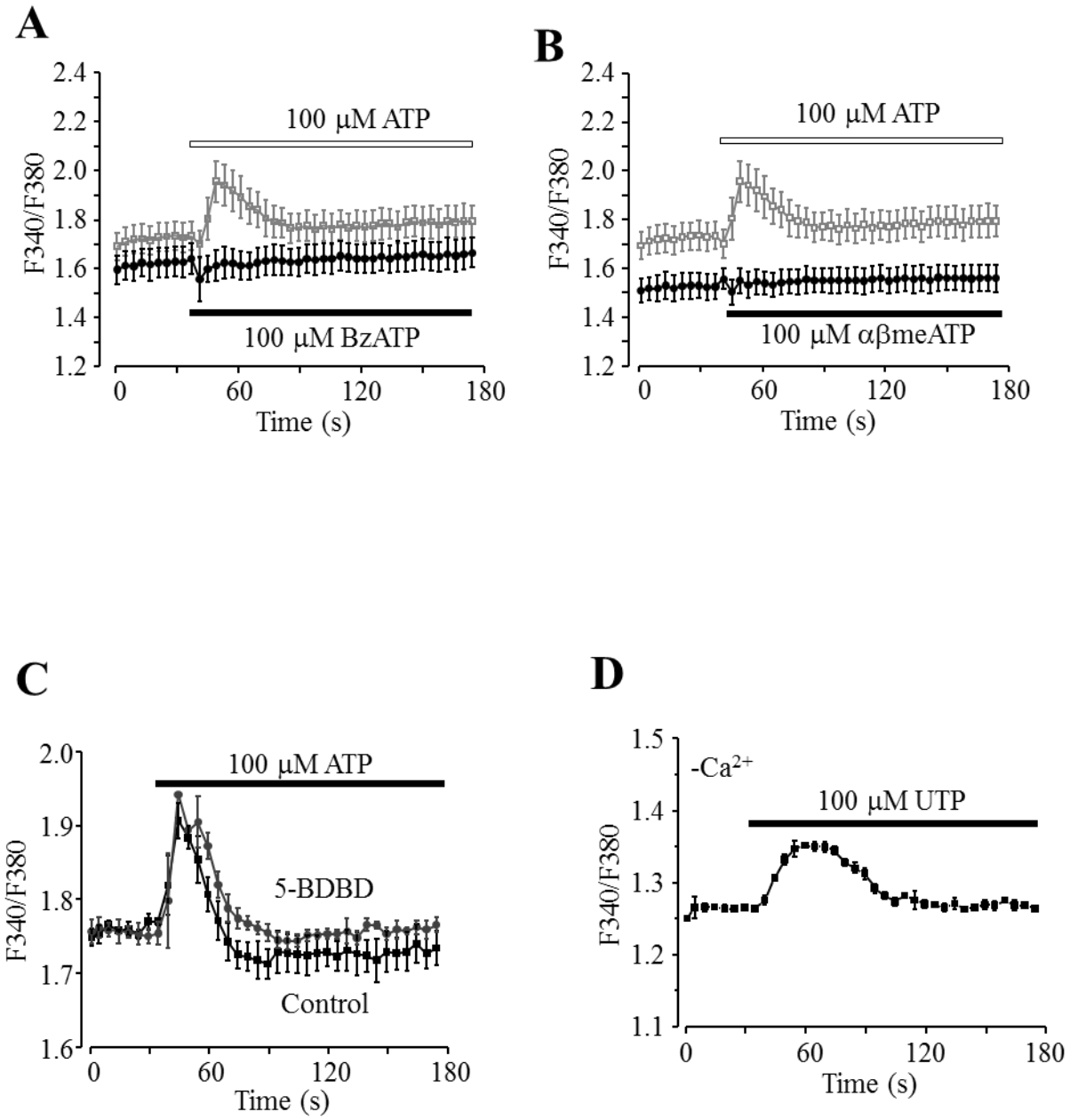
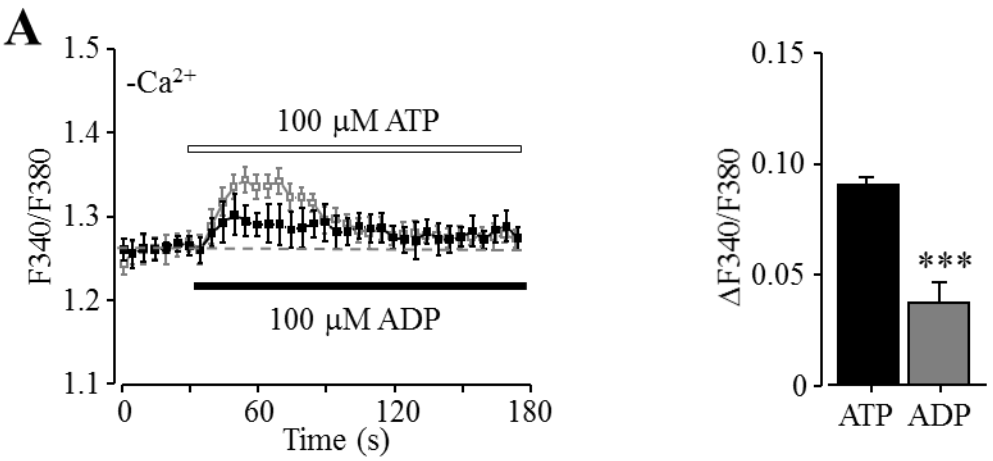


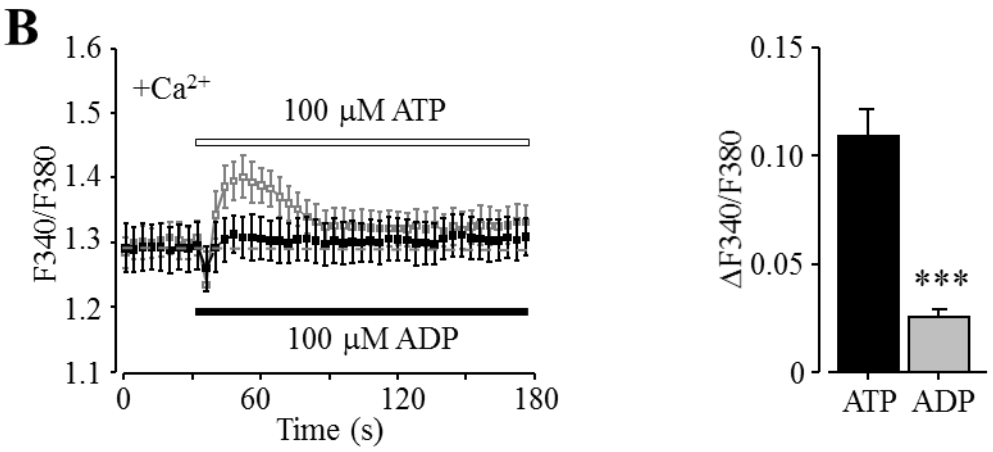
Fig 4.5 Ca^{2+} responses to P2X or P2Yagonists in Huh-7 cell.

(A-B) Representative recordings of the Ca^{2+} responses induced by 100 μM BzATP (A) and 100 μM αβmeth ATP (B) as well as 100 μMATP (grey). Such results were observed in three independent experiments. (C) Representative recordings of 100 μM ATP-induced Ca^{2+} responses in control cells or cells pretreatment of 10 μM 5-BDBD. (D) Representative recordings of the Ca^{2+} responses caused by 100 μM UTP in Ca^{2+}-free solutions extracellularly.

4.7 The P2Y receptor role in ATP-induced increase in the $[Ca^{2+}]$

We further determined the role of P2Y receptors in Huh-7 cells after determining the role of P2XR in Huh-7 cells. There was no noticeable expression of P2X receptors in Huh-7 cells. P2Y receptors in ATP-induced increase in the $[Ca^{2+}]$ was examined in the extracellular Ca^{2+}-free solution. A widely-used experimental condition to determine Ca^{2+} release from internal stores. There was significant increase in the $[Ca^{2+}]_i$ in the extracellular Ca^{2+}-free solutions, clearly supporting presence of the P2Y receptors functionally , although the amplitude of ATP-induced Ca^{2+} response in the extracellular Ca^{2+}-free solutions was lower than that in the extracellular Ca^{2+}-containing solutions (Fig 4.6A). To elaborate which particular P2Y receptor is most important in mediating ATP-induced Ca^{2+} response, we further examined several P2Y selective agonists. Exposure to 100 μM ADP, an agonist that prefers to activate the P2Y1 receptor, induced much smaller Ca^{2+} responses than ATP in both extracellular Ca^{2+}-free and Ca^{2+}-containingsolutions (Fig 4.6B and 4.6C). Moreover, application of 10 nM MRS2365, a selective P2Y1receptor agonist, was ineffective in inducing an increase in the $[Ca^{2+}]_i$(Fig 4.6D). Theseresults consistently indicate no major role for the P2Y1 receptor in ATP-induced Ca^{2+}response.


A
-Ca2+
100 µM ATP
100 µM ADP
F340/F380
Time (s)
ΔF340/F380
***
ATP
ADP


B
+Ca2+
100 µM ATP
100 µM ADP
F340/F380
Time (s)
ΔF340/F380
***
ATP
ADP

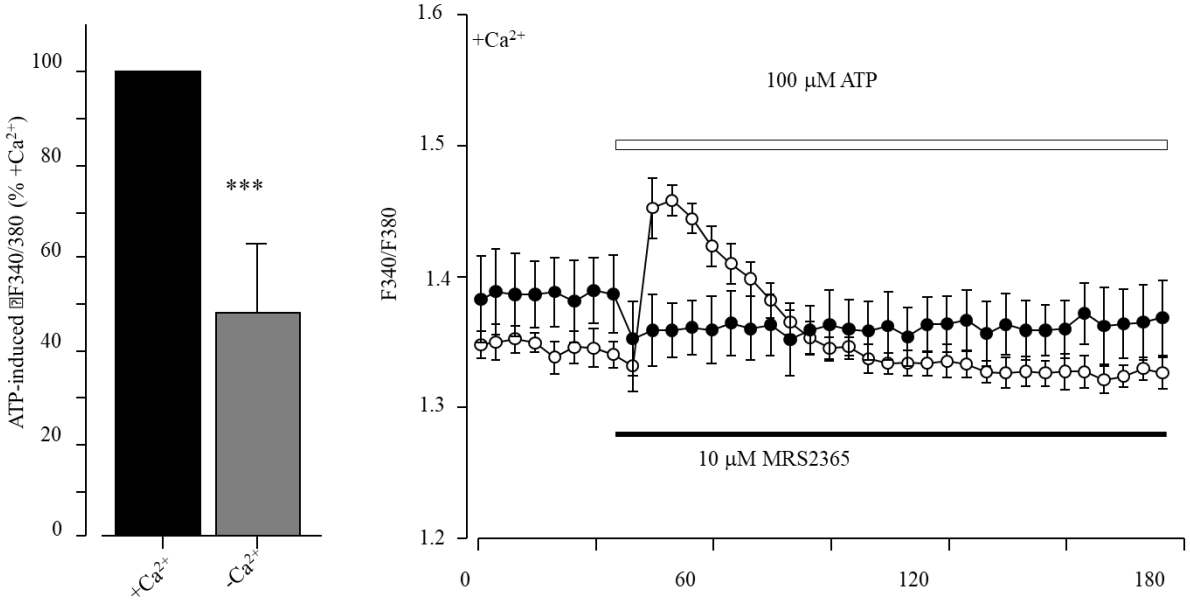
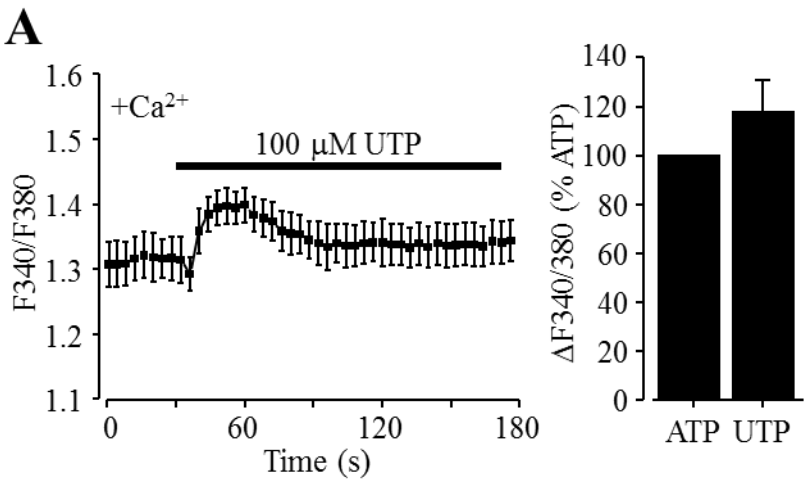
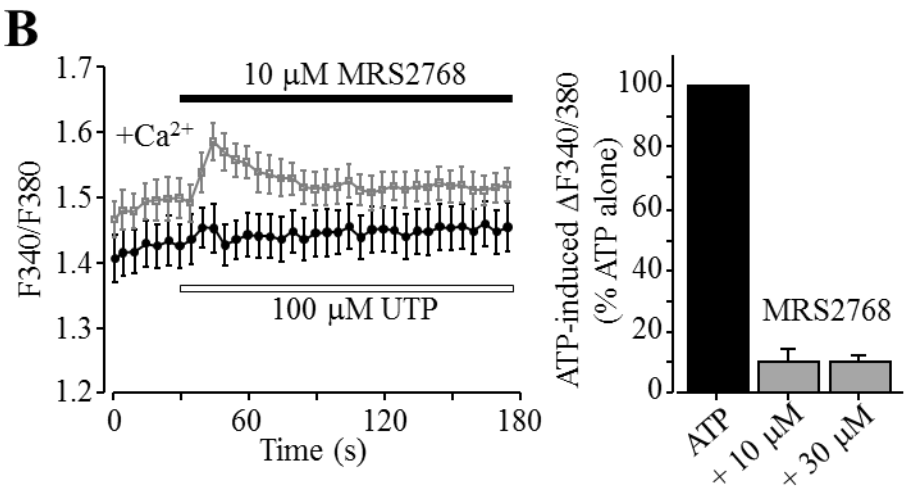


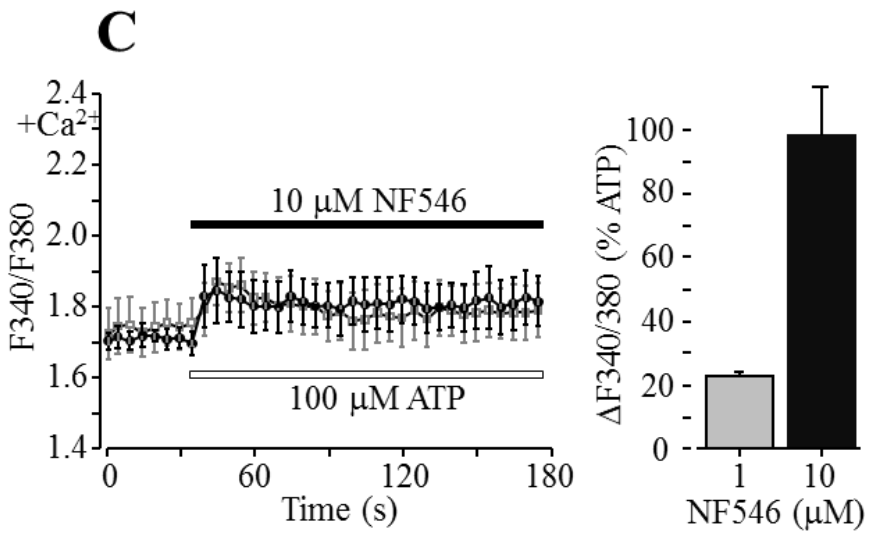
Fig 4.6 Role of the P2Y1 receptor in ATP-induced increase in the $[Ca^{2+}]$i in Huh- 7 cells.

(A) *Left*, illustrative records of the Ca^{2+} responses induced by 100 μM ATP (grey) and ADP (black) in the extracellular Ca^{2+}-free solutions. *Right*, summary of the peak increase in the $[Ca^{2+}]_i$. (b) *Left*, representative recordings of the Ca^{2+} responses induced by 100 μM ATP (grey) and ADP (black) in the extracellular Ca^{2+}-containing solutions *Right*, summary of the peak increase in the $[Ca^{2+}]_i$. (c) Representative recordings of the Ca^{2+} responses induced by 100μM ATP (grey) and 10 μM MRS2365 (black) in the extracellular Ca^{2+}-containing solutions. ***, $p < 0.001$.

UTP is a specific agonist at the $P2Y_2$ receptor as well as at the $P2Y_4$ and $P2Y_6$ receptors, found to be equally effective as ATP in uplifting the $[Ca^{2+}]_i$ in the extracellular Ca^{2+}-containing solutions (Fig. 4.7A), as previously reported [39]. UTP was also effective in inducing internalCa^{2+} release as shown in the extracellular Ca^{2+}-free solutions (Fig 4.5D). Thesefindings are constant with the pharmacological properties of the P2Y2 receptor, previously shown to be expressed in both human hepatocytes and HCC cells [38, 39]. Accordingly, it wassurprising to find that exposure to 10 and 30 uM MRS2768, a selective $P2Y_2$ receptor agonist, failed to elevate the $[Ca^{2+}]_i$ in the extracellular Ca^{2+}-containing solutions (Fig 4.7B). Thisobservation was made using two batches of MRS2768 from different vendors (see Materialsand Methods). Application of 1 and 10 uM NF546, a selective P2Y11 receptoragonist, concentration-dependently increased the $[Ca^{2+}]_i$ in the extracellular Ca^{2+}-containingsolutions, and the Ca^{2+} response amplitude induced by 10 uM NF546 was virtually the sameas that induced 100 uM ATP (Fig 4.7C). $P2Y_{11}$ receptor expression in huh-7 cells demonstrated through Immunocytochemistry (Fig 4.11B). Furthermore, pre-treatment with 10uM NF340, a selective P2Y11 receptor antagonist, almost completely abolished ATP stimulated elevation of the $[Ca^{2+}]_i$ in the extracellular Ca^{2+}-containing solutions (Fig 4.7D). Taken together, our results provide consistent functional indication of $P2Y_{11}$receptor in mediating ATP-induced Ca^{2+} response in Huh-7 cell


A
+Ca2+
100 µM UTP
F340/F380
Time (s)
ΔF340/380 (% ATP)
ATP
UTP


B
10 µM MRS2768
+Ca2+
100 µM UTP
F340/F380
Time (s)
ATP-induced ΔF340/380
(% ATP alone)
MRS2768
ATP
+ 10 µM
+ 30 µM


C
2.4
+Ca2+
2.2
2.0
1.8
1.6
1.4
F340/F380
10 μM NF546
100 μM ATP
0
60
120
180
Time (s)
ΔF340/380 (% ATP)
100
80
60
40
20
0
1
10
NF546 (μM)

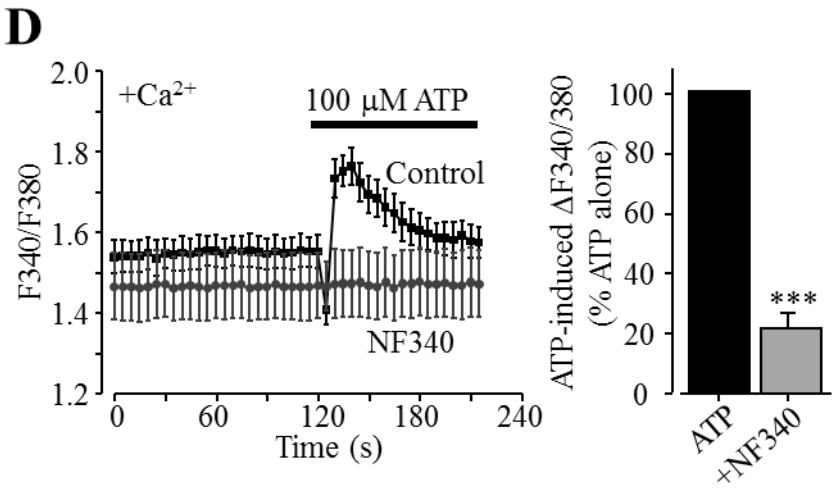

D
2.0
+Ca2+
100 μM ATP
1.8
Control
1.6
1.4
NF340
F340/F380
1.2
0
60
120
180
240
Time (s)
ATP-induced ΔF340/380
(% ATP alone)
100
80
60
40
20
0
***
ATP
+NF340

Fig 4.7 $P2Y_{11}$ receptor role in ATP-induced increase in the $[Ca^{2+}]_i$ in Huh-7 cells.

(A) *Left*, illustrative records of the Ca^{2+} responses stimulated with by 100 μM UTP in the solutions with Ca^{2+} extracellularly. *Right*, summarized aboveUTP-induced peak increase in the $[Ca^{2+}]_i$, it is expressed as % of that induced by 100 μMATP, all readings are recorded from eighteen wells of cells in three independent experiments. (B) *Left*, illustrativerecords of the Ca^{2+} responses induced by 10 μM MRS2768 (black) and 100 μM ATP (grey) in the extracellular Ca^{2+}-containing solutions *Right*, summary of UTP-induced peak increases in the $[Ca^{2+}]_i$, expressed as % of that inducedby 100 μM ATP, (C) *Left*,representative recordings of the Ca^{2+} responses induced by 10 μM NF546 (black) and 100μM ATP (grey) in the extracellular Ca^{2+}-containing solutions. *Right*, summary of the peak increases in the $[Ca^{2+}]_i$ induced by 1 and 10 μM NF546. It is expressed as % of that induced by 100 μM ATP (D) *Left*, representative recordings of the Ca^{2+} response producedwith 100 μM ATP in control cells or cells pre-treatment of 10 μM NF340 *Right*, summary of ATP-induced peak increases in the $[Ca^{2+}]_i$in control orNF340- treated cells, expressed as % of that in control cells. ***, $p < 0.001$.

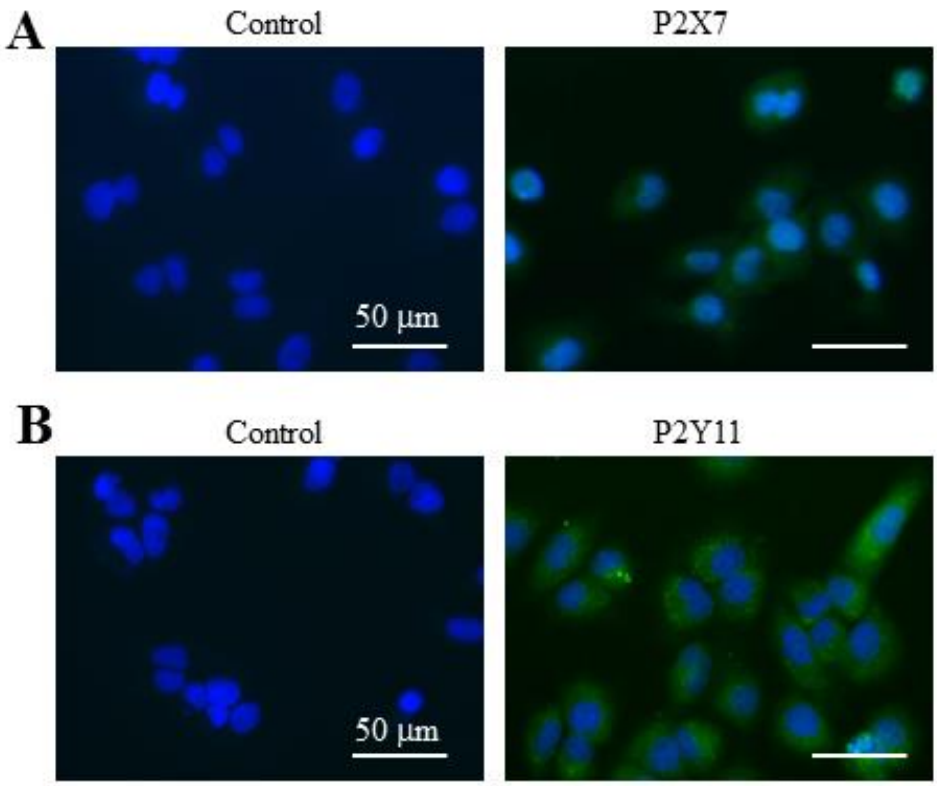
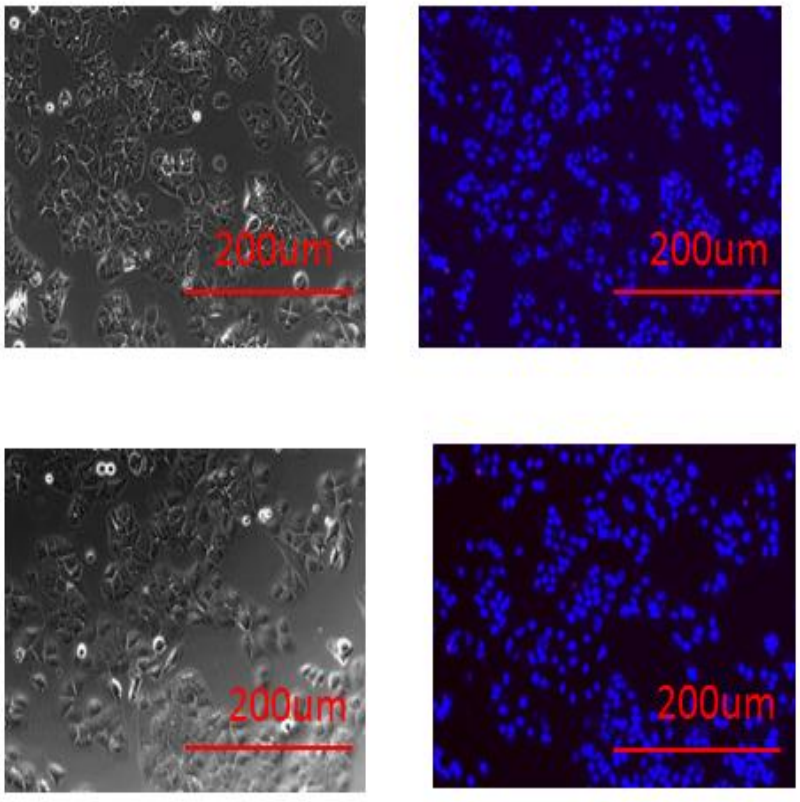


Fig 4.8 Immunostaining of the P2X7 and P2Y11 receptors in Huh-7 cell.

Representative confocal images showing control cells stained only with the secondary antibody and cells stained with primary anti-P2X7 (A) and anti-P2Y11 antibody (B) and the secondary antibody. Cells were count-stained with DAPI. Similar results were observed in two independent experiments.

4.8 ATP has no detrimental effect on Huh-7 cells

Huh7 cells were grown overnight and pre-treated with 100µM of ATP and for control only media was added. And after 24 hrs. And cells were treated with PI and Hoechst staining under control conditions. There was no dead cells in the media. This confirms that ATP does not have detrimental effects on Huh-7 cells after they were treated for overnight. Experiments were repeated in triplicate and results were imaged using EVOS® Cell Imaging System (Fig 4.9).



A. Control

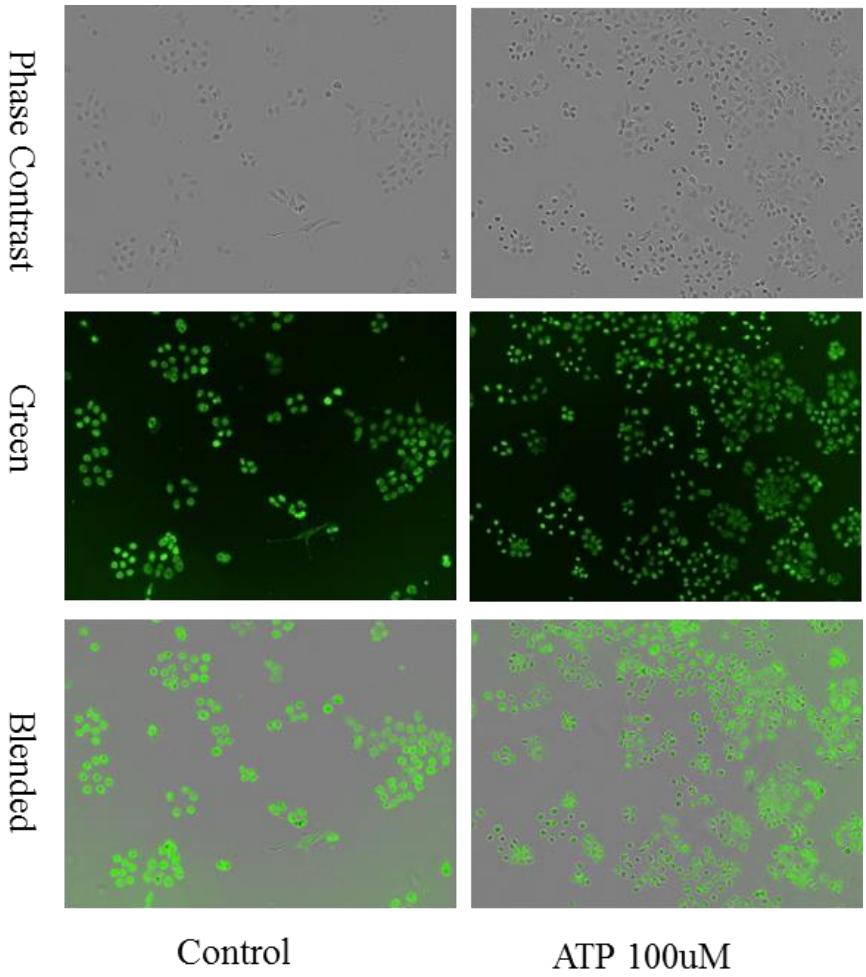
B. ATP

Fig. 4.9 Effect of ATP on Huh-7 cell survival.

Representative bright field (left) and merged images (right) showing PI and Hoechst staining under control conditions (A) and in the presence of 100 µM ATP for 24 hr (B) from four wells of cells for each condition.

4.9 Effect of different agonist and antagonist on cell proliferation

Huh-7 cells were incubated with ATP and effect on cell proliferation was measured from 0hr, 24hr, 48hr, and 72hr by adding syber green dye. Cell with no ATP were used as control. There was an increase in the proliferation rate of Huh-7 cells in the presence of ATP at 48hr and 72hr (Fig. 4.10a). This give us a clue that ATP might have a role in Huh-7 cell proliferation. NF546, A $P2Y_{11}$ selective agonist, was used to test the effect on Huh-7 cell proliferation. NF546 was used at 10µM and 100µM conc. to incubate Huh-7 cells and effect on proliferation was measured at 0hr, 24hr, 48hr, and 72hr. There was a noticeable change in huh-cell proliferation rate at 72hrs with 100µM conc. of NF546 (Fig.4.10b). Next step was to measure the effect of NF340, a selective antagonist for $P2Y_{11}$ receptor, on Huh-7 cell proliferation. Effect of NF340 (10µM) inhibition was measured by incubating the Huh-7cells alone with NF340 and ATP+NF340. There was a significant inhibition by NF340 (10µM) when cell were incubated with both ATP+NF340 at 48hr and 72hr (4.10c).


Phase Contrast
Green
Blended
Control
ATP 100uM

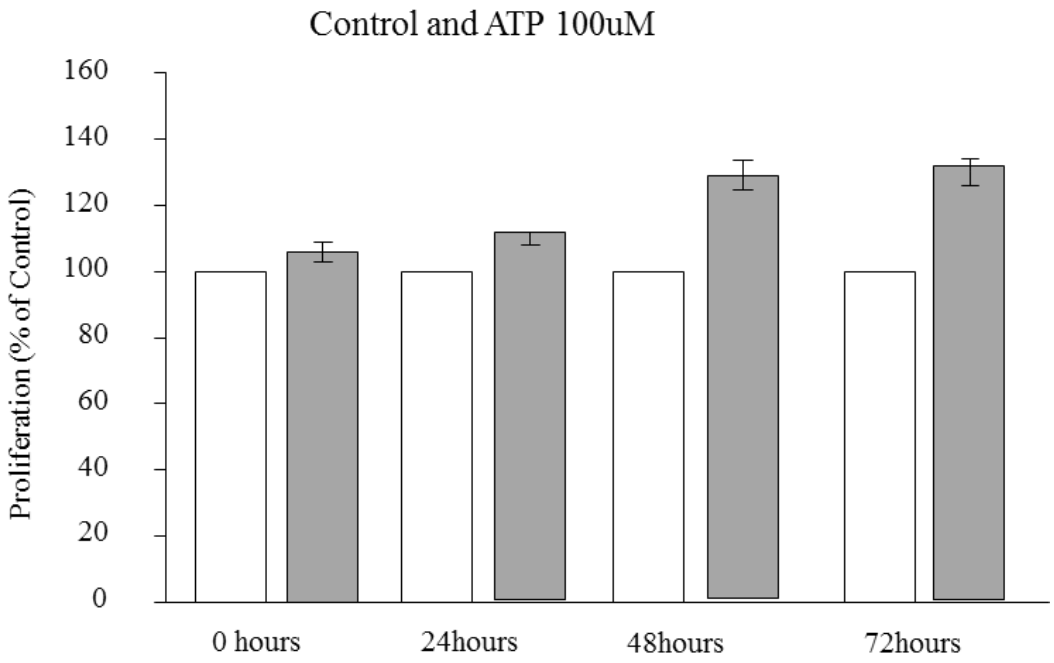
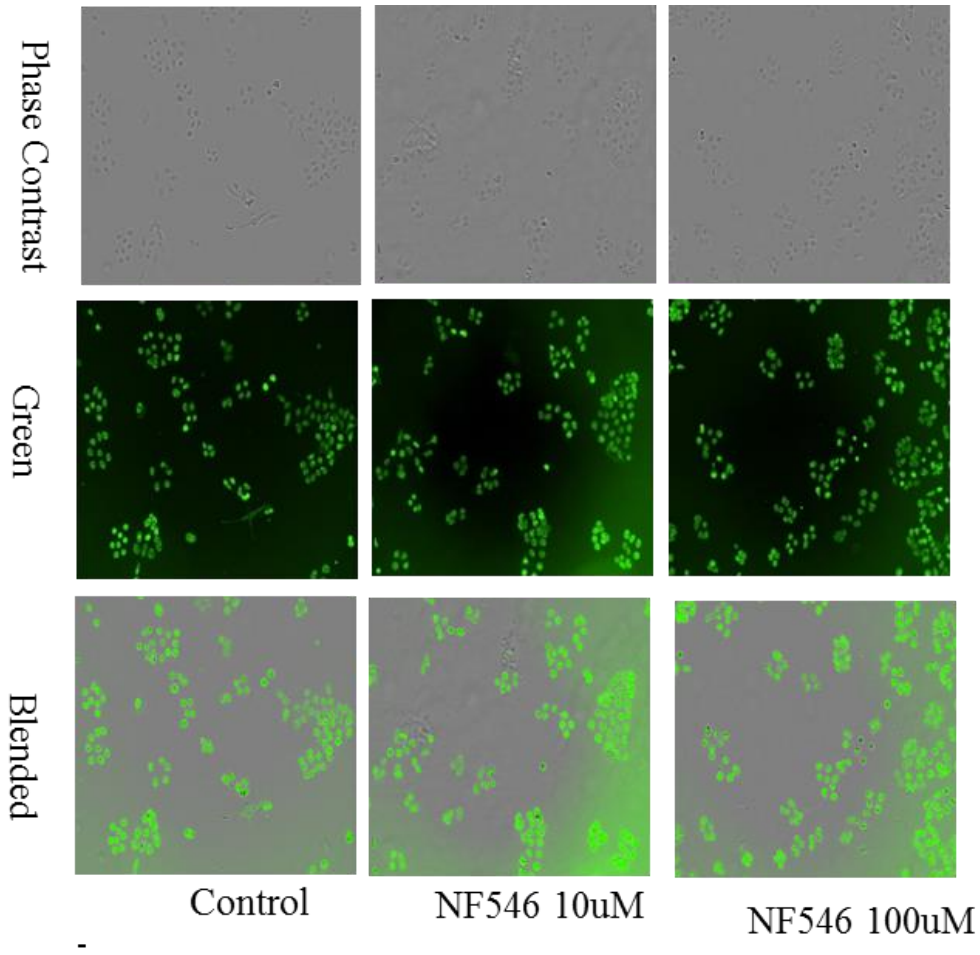


Fig 4.10A Huh-7 cell proliferation.

Right Representative images showing huh7 cell proliferation images taken after 72hrs of treatment with 100µM ATP. Syber Green dye was added to the cells to capture Images from live huh7 cells. Left Bar graph shows Huh-7 cells proliferation after 0, 24, 48, 72 hrs. of treatment with ATP.


Phase Contrast
Green
Blended
Control
NF546 10uM
NF546 100uM

-

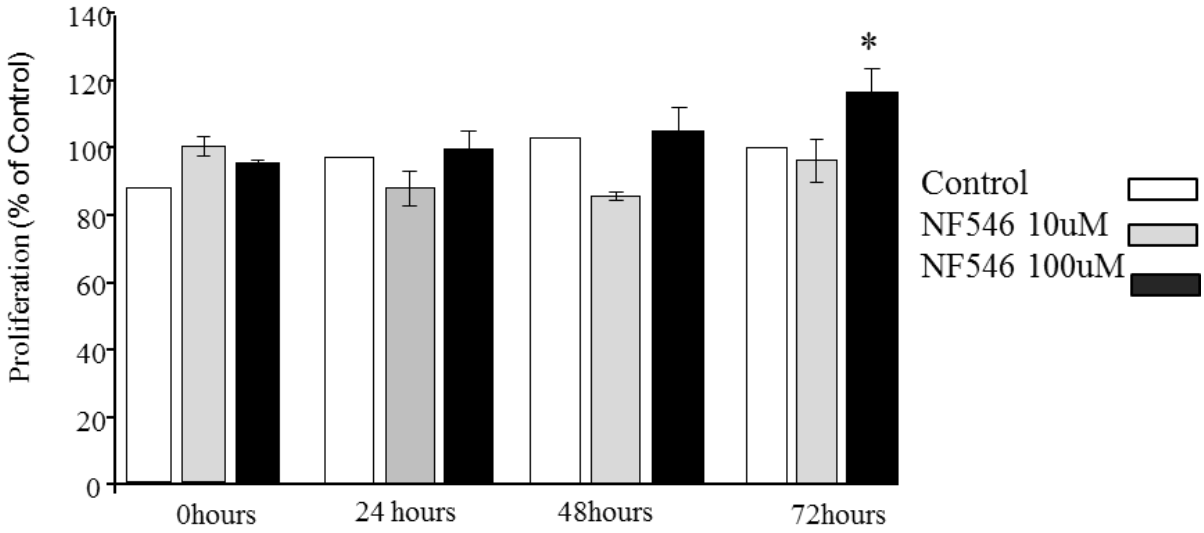
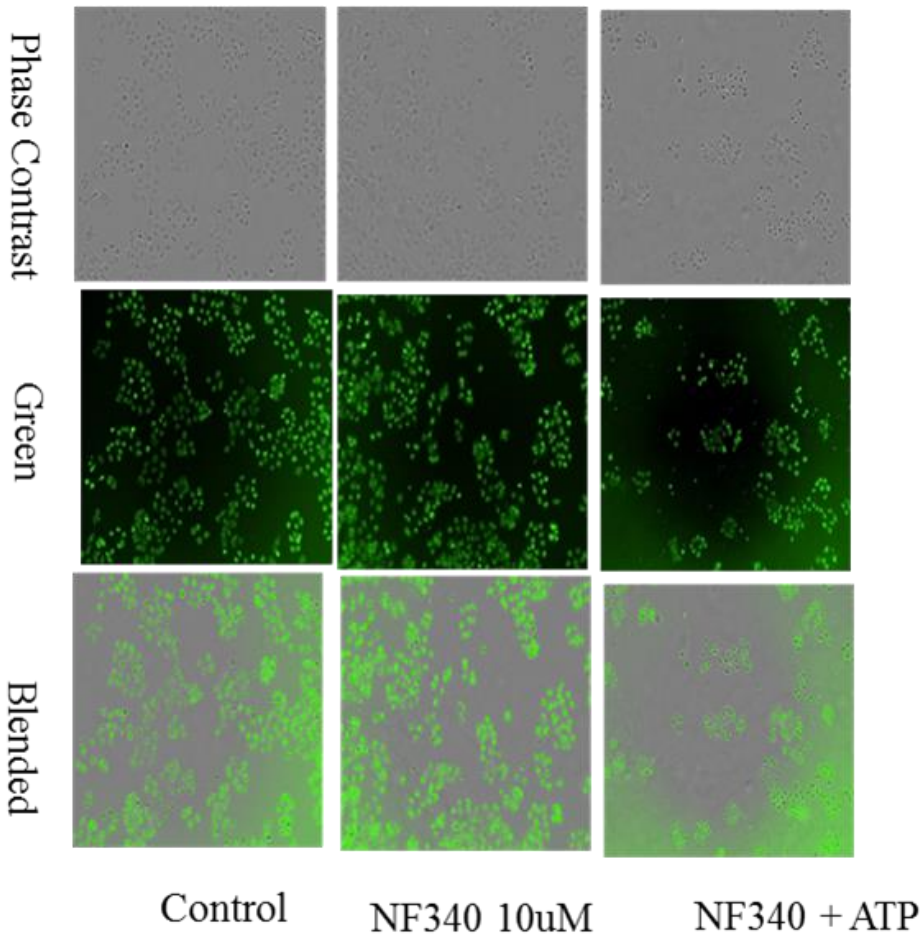


Fig 4.10B Huh-7 cell proliferation.

Top Representative Images showing huh7 cell proliferation images taken after 72hrs of treatment with 100µM ATP, 10µM NF546, and 100µM NF546. Syber Green dye was added to the cells to capture Images from live Huh-7 cells. Bottom Bar graph shows huh7 cells proliferation after 0, 24, 48, 72 hrs of treatment with ATP, NF546 (10,100 µM).


Phase Contrast
Green
Blended
Control
NF340 10uM
NF340 + ATP

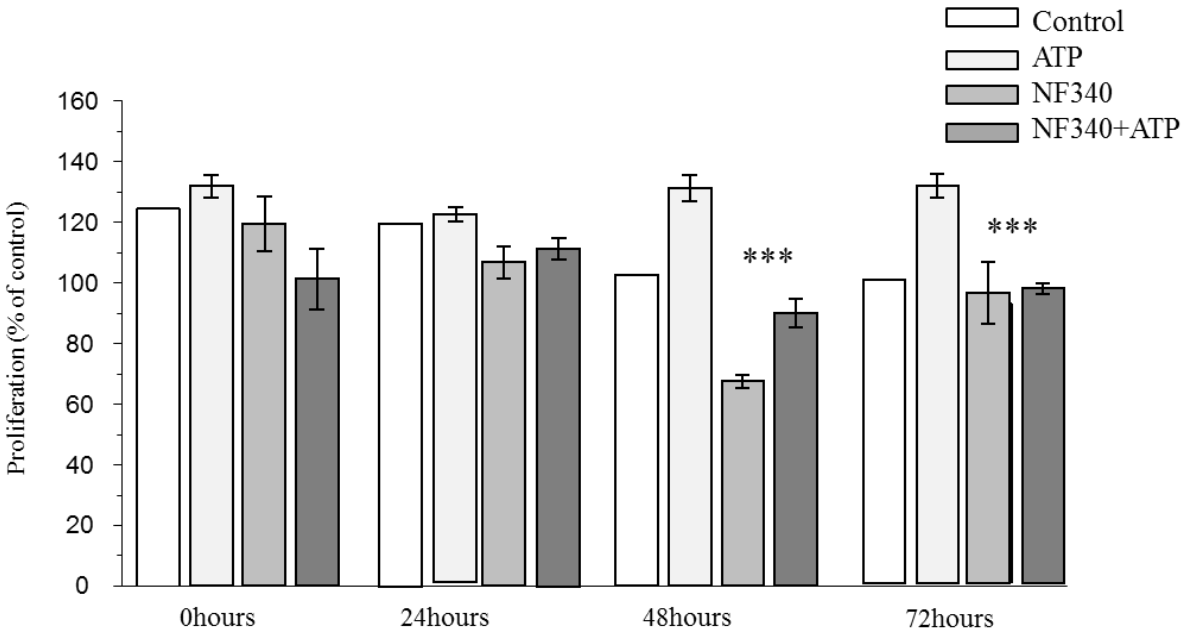


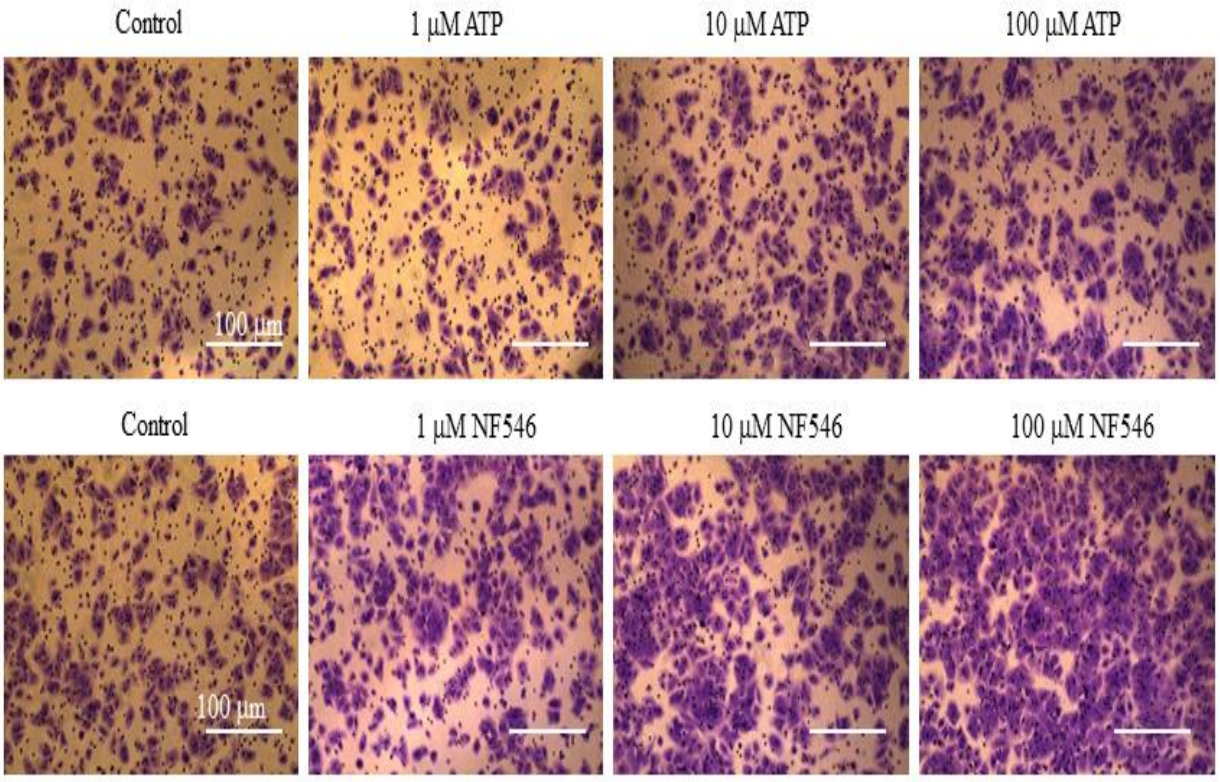
Fig 4.10C Huh-7 cell proliferation.

Top Representative Images showing huh7 cell proliferation images taken after 72hrs of treatment with 100uM ATP, 10uM NF5340, and NF340+ATP (10µM, 100µM). Syber Green dye was added to the cells to capture Images from live Huh-7 cells. Bottom Bar graph shows huh7 cells proliferation after 0, 24, 48, 72 hrs of treatment with ATP, NF340, NF340+ATP (10,100 µM).

4.10 ATP stimulates Huh-7 cell migration via the $P2Y_{11}$ receptor

ATP has been shown to promote cell migration of intrinsic human HCC cells and in *in vitro* cells specific for liver cancers. Activation of the $P2Y_2$ receptor is proposed to be critical in mediating ATP-induced cell migration. This current study did not examine $P2Y_{11}$ receptor expression including ATP-induced stimulation of HCC cell migration. We thus performed the trans-well chamber assay to examine extracellular ATP stimulated Huh-7 cell migration and if so, whether the $P2Y_{11}$ receptor has a role. Cells with no ATP was used as control. ATP applied at 1, 10 and 100 µM resulted in concentration dependent stimulation of cell migration (Fig 4.11A). The increase in cell migration by 10 and100 µM ATP reached the significant level (Fig 4.11C). Treatment with 1, 10 and 100 µMNF546 also stimulated cell migration with varying concentration (Fig 4.11B and D).At the same concentrations (i.e., 10 or 100 µM), NF546 induced slightly greater increase in cell migration than ATP (Fig 4.11 C and D). Treatment with ATP at 100 µM induced no detectable cell death (Fig 4.9). Collectively, these results support the presence of $P2Y_{11}$ receptor and its role in driving ATP stimulated migration of Huh-7 cells. We also examined the effect of $P2Y_{11}$ receptor antagonist NF340 on ATP stimulated migration of Huh-7 cells. 10 µM NF340 completely blocked ATP stimulated migration of Huh-7 cells with no significant effect on cell migration under the basal condition, providing further evidence to support that $P2Y_{11}$ receptor activation is critical in stimulating cell migration by ATP (Fig 4.12 A and B). It is well known that ATP is metabolically unstable and can be rapidly metabolized to ADP and further to adenosine byecto-enzymes. Adenosine also acts as an extracellular signaling molecule via activating

G-protein-coupled adenosine receptors on the cell surface. Activation of the adenosine receptors has been shown in a very recent study to be significantly involved in ATP-induced regulation of breast cancer cell migration. ATP induced stimulation of HCC cell migration has not been investigated in previous studies with reference to adenosine receptors. Therefore, we examined the effect of CGS15943, a generic antagonist for adenosine receptors, on ATP-derived stimulation of Huh-7 cell migration. Intriguingly, treatment with 100 nM CGS15943 was also effective in preventing ATP-induced increase in cell migration with no significant effect on cell migration under the basal condition (Fig 4.12 A and C), suggesting a substantial role for adenosine receptors in ATP-induced stimulation in HCC cell migration.


Control
1 µM ATP
10 µM ATP
100 µM ATP
100 µm
Control
1 µM NF546
10 µM NF546
100 µM NF546
100 µm

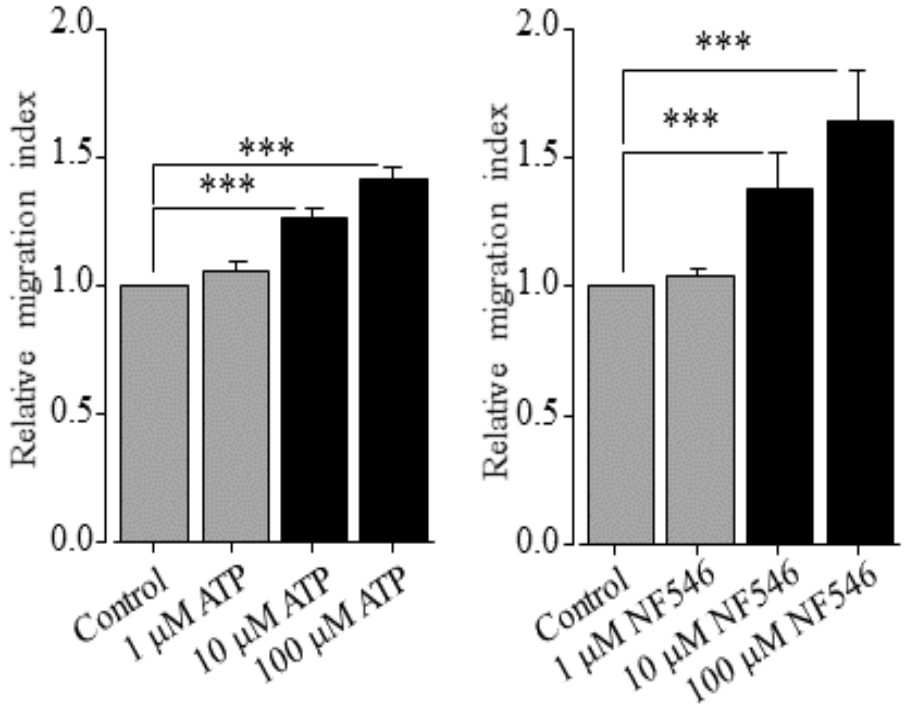
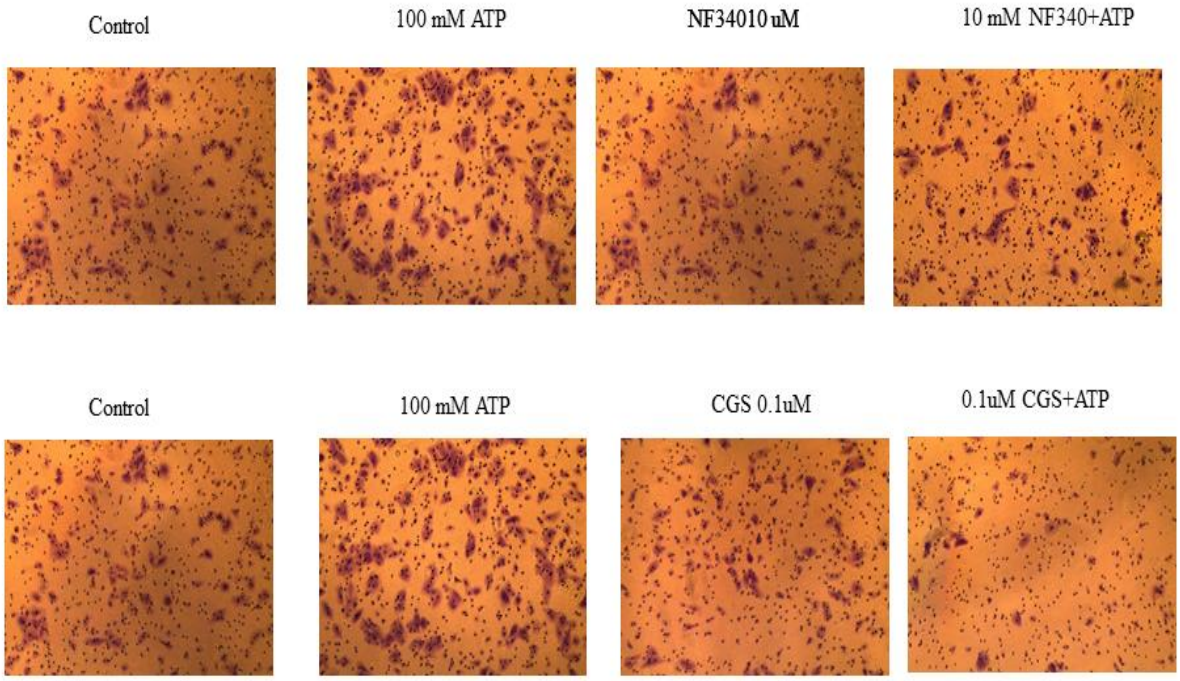


Fig 4.11 Outcomes of ATP on Huh-7 cell migration through P2Y11 receptor activation.

(A-B) Typical images of crystal violet staining show cell migration under basal condition. And designated concentrations of ATP (A) and NF546 (B) in trans-well assays. (C-D) Summarized view of the effects of different concentrations of ATP (C) and NF546 (D) on cell migration. All are representative of three independent experiments, respectively. ***, $p < 0.001$.


Control
100 mM ATP
NF34010 uM
10 mM NF340+ATP
Control
100 mM ATP
CGS 0.1uM
0.1uM CGS+ATP

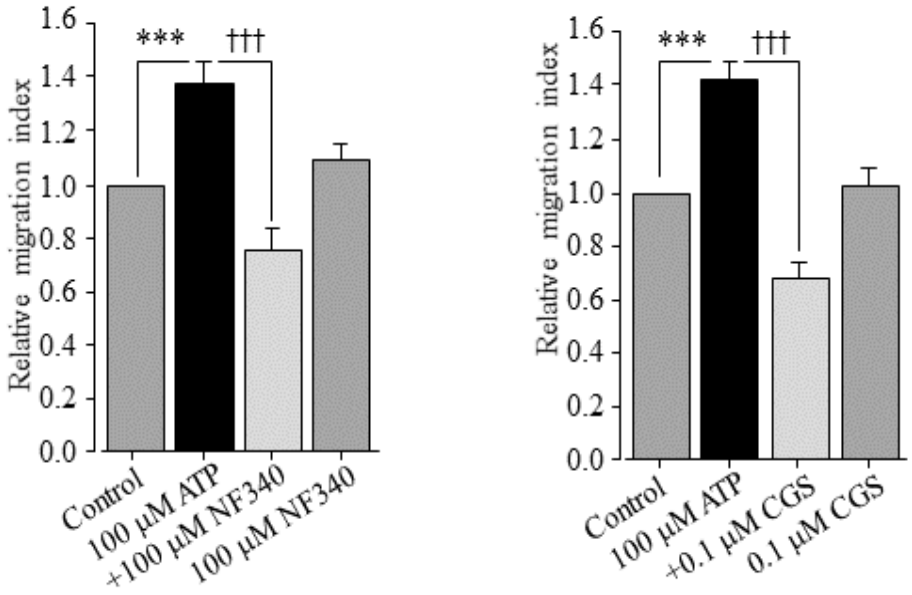


Fig 4.12 $P2Y_{11}$ receptor and adenosine receptor involvement in ATP-stimulated Huh-7 cell migration.

(A) Archetypal images with crystal violet staining showing cell migration under basal condition and in the presence of 100 μM ATP alone, 10 μM NF340 alone, 100 nMCGS15943 alone, and 100 μM ATP and 10 μM NF546 (ATP+NF340), 100 μM ATP and 100nM CGS15943 (ATP+CGS) in trans-well assays. (B-C) Summarized effects of treatment with NF340 (B) and 100 nM CGS15943 (C) on ATP-induced increase in cell migration fromthree independent experiments. ***, †††, $p < 0.001$.

4.11 Simulation and Modeling

This part of study readily focuses on GPCR-associated calcium signaling with mutated downstream signaling proteins. Moreover quantitative modeling of the GPCR-associated signaling will be done and specific agonist and antagonist will be used as nodes with specific values to activate and inhibit the calcium signaling, results obtained will completely mimic our previous wet lab studies.

GPCR-associated signaling network in HCC was studied at diverse data points that are interlinked on the basis of previous studies performed.

i. Extracellular ATP-induced Ca^{2+} signaling is critical in regulating diverse physiological and disease processes including cancer cell functions. ATP activates G-protein coupled P2Y receptors that leads to release of Ca^{2+} from intracellular stores via $G_{\alpha,q/11}$-PLC-IP_3R signaling pathway(Madiha *et al.,* 2017)

ii. At the cellular level, AKT/Ras co-activation resulted in increased proliferation and angiogenesis when compared with AKT mice, leading to rapid malignant transformation and tumor progression. (Chunmei *et al.,* 2013)

iii. *TP53 mutations in DNA* binding domain of p53 resulting in a lower affinity to bind the sequence-specific response elements of its target genes occur in HCC. This also decreases p53-mediated induction of *MDM2.* Consequently, the misregulation of MDM2 results in elevated levels of mutant p53 in many tumors cells or tissues (Xuan Meng *et al., 2015).* Finally, the BRN was constructed on the basis of constitutive GPCR receptor activation while binding with ATP as growth factor that leads towards activation of downstream signaling proteins with loss of function mutation in P53.

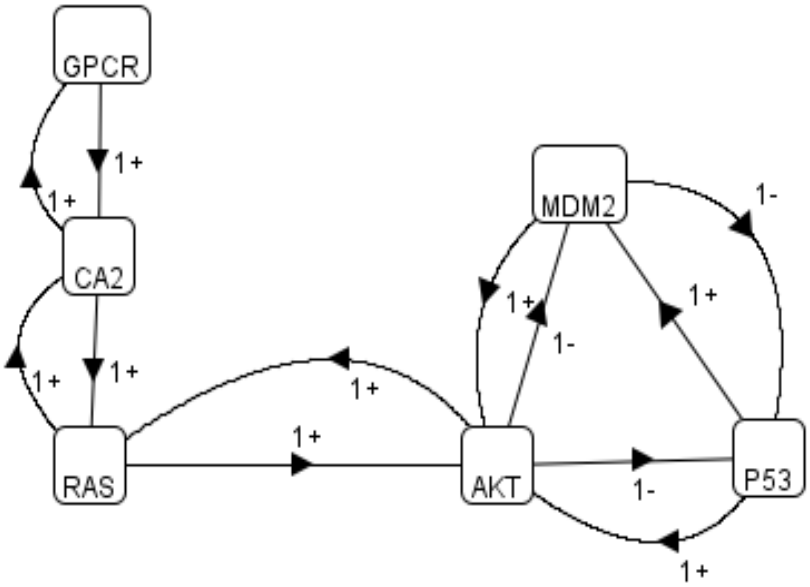


Fig. 4.13 GPCR- associated BRN.

Arrows are used to indicate the activation and inhibition. A positive (+) sign indicated activation and a negative (-) sign indicate inhibition. The formal description of the BRN is N= {GPCR, CA2, RAS, AKT, MDM2, P53}; ED= {(GPCR→CA2), (CA2→RAS), (RAS→AKT), (AKT→MDM2), (AKT→P53), (P53→MDM2), (MDM2→AKT), (MDM2→P53), (P53→AKT)}

4.12 Analysis of GPCR- Associated BRN

To generate the state graph shown in Fig 4.16 and for analysis of BRN, a tool named as GENOTECH was used. The baseline state is at (0,0,0,0,0,0) and the metastatic deadlocked state is at (1,1,1,1,0,0). To describe the state graph completely, there were 63 states, 23 unique cyclic trajectories between these states. These cycles represent the effect of participating entities expression level on each other. State graph exhibited a set of cycles that were extracted from the selected parameter of biological observations. These findings led us to separate different trajectories arising from this BRN leading towards liver cell migration with up-regulated expression of GPCR, Ca^{2+}, Akt, Ras. Activation of GPCRs after binding with ATP leads to downstream activation of calcium and other signaling proteins. Binding of IP3 with Akt moves the cell towards cell survival. Continuous sensitization of GPCR causes intracellular influx of calcium that can keep Akt as activating and suppression of tumor suppressor genes can lead towards cell proliferation and migration in cancer cells. Since calcium levels are high in huh7 and HepG2 cell lines when exposed to different ATP concentrations. This high levels of ATP can keep cell in survival state with suppression of tumor suppressor genes as observed in final cycles where receptor is activated and calcium levels are high. This can ultimately lead to deadlock state that is the proliferation of liver cells (Fig 4.17).

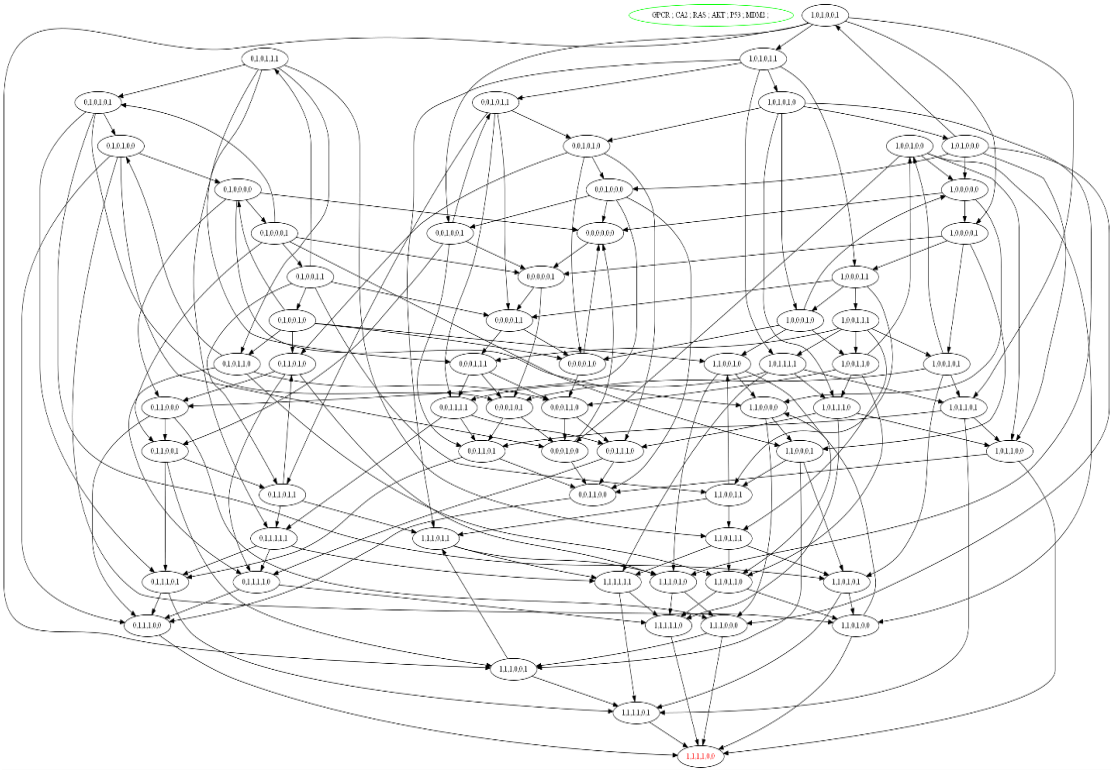


Figure 4.14 GPCR_ associated BRN in HCC.

All the logical parameters were constructed by using the tool GENOTECH. Baseline is taken as zero which is considered as suppressed state with no expression (0,0,0,0,0) whereas the deadlocked state (1,1,0,0,0) represents the metastatic state where only GPCR, CA, RAS and Akt remain persistently active. In cancerous state GPCR, CA, RAS and Akt remain active while p53, Mdm2 genes are under constant suppression

Table 4.2 List of discrete parameters of each entity of the BRN.		
Sr. No.	Biological entities	Discrete parameters
1.	GPCR	K (GPCR , { }) = 0 K (GPCR , { CA2 }) = 1
2.	CA2	K (CA2 , { }) = 0 K (CA2 , { RAS }) = 1 K (CA2 , { GPCR }) = 1 K (CA2 , { GPCR , RAS }) = 1
3.	RAS	K (RAS , { }) = 0 K (RAS , { AKT }) = 1 K (RAS , { CA2 }) = 1 K (RAS , { CA2 , AKT }) = 1
4.	AKT	K (AKT , { }) = 0 K (AKT , { MDM2 }) = 1 K (AKT , { P53 }) = 1 K (AKT , { P53 , MDM2 }) = 1 K (AKT , { RAS }) = 1

		K (AKT , { RAS , MDM2 }) = 1 K (AKT , { RAS , P53 }) = 1 K (AKT , { RAS , P53 , MDM2 }) = 1
5.	P53	K (P53 , { }) = 0 K (P53 , { AKT }) = 1 K (P53 , { MDM2 }) = 0 K (P53 , { AKT , MDM2 }) = 0
6.	MDM2	K (MDM2 , { }) = 0 K (MDM2 , { P53 }) = 0 K (MDM2 , { AKT }) = 1 K (MDM2 , { AKT , P53 }) = 0

.

4.13 Effects of Simulation on GPCR Signaling Model

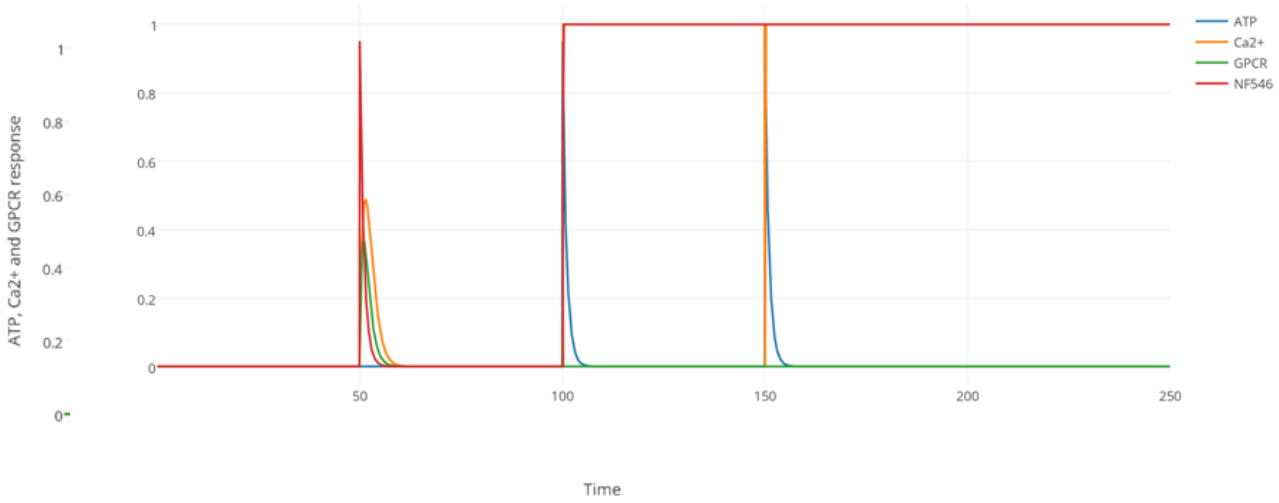
We inferred the regulatory network controlling the calcium signaling from published experimental data. Network included nodes and regulatory interactions for activators/inhibitors. There are five nodes in the network; namely, $P2Y_{11}$, Ca^{2+}, ATP, NF546, NF340. These nodes have nine regulatory interactions among them, being either positive (i.e., activatory), or negative (i.e., inhibitory). Under normal physiological conditions P2Y11, a G-protein coupled receptor, acts as an on-off switch with numerous physiological and pathological responses. Purinergic receptors comprise a family of transmembrane receptors, which are activated by extracellular nucleosides and nucleotides.

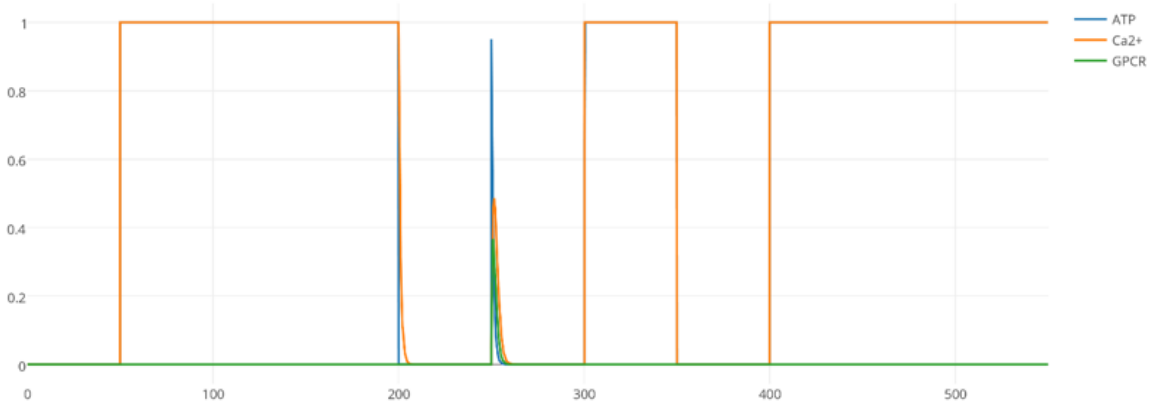
In this case effects of variable doses of activators and inhibitors were tested on the normal levels of GPCR, ATP and Ca^{2+} to confirm the activity of the receptor in downstream Ca^{2+} signaling. Normal levels are assigned zero value for node at normal physiological conditions and decay value of 1. Drugs applied at higher concentration of (100µm) with node values assigned as 1 and decay value of 0.001.

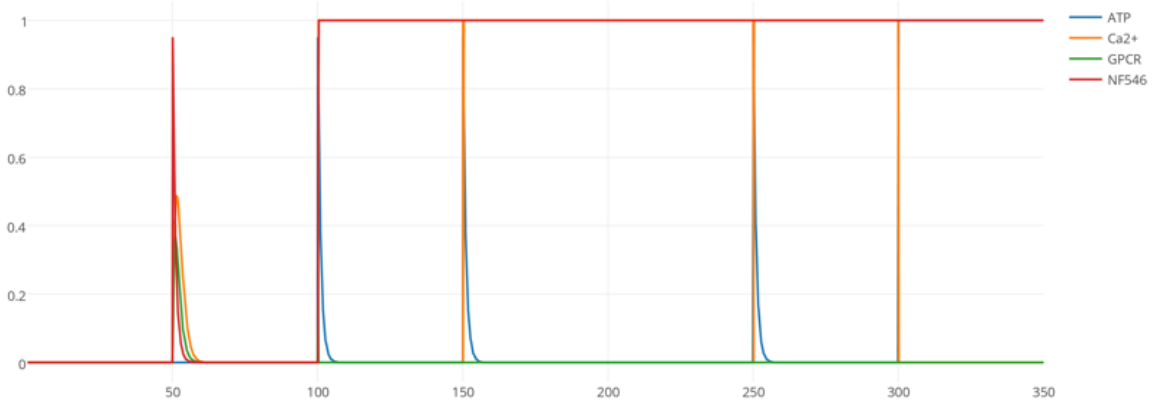
To examine the role of $P2Y_{11}$ receptors in ATP-induced increase in the $[Ca^{2+}]_i$, we determined ATP-induced Ca^{2+} response initially at minimum conc. of ATP. In this case node values assigned as 0 for ATP, $P2Y_{11}$ and Ca^{2+} with decay factor of 1 shown in table 1. Both GPCR and Ca^{2+} are show in normal state and ATP is supposed to add at minimal concentration of 1µM in the system to observe the change in Ca^{2+} response. There was an increased response of Ca^{2+} when ATP was added in the system, but it drops down suddenly after the ATP is decayed after 300 sec. to obtain optimum dose response model was given with high con. of ATP (100µM). High conc. was assigned node value of 1. There was a robust response in the Ca^{2+} response that became sustained after some time.

Our next target was to use the activator drug NF546 that can mimic the ATP induced- Ca^{2+} response in the same manner. NF546 was added at the conc. of 10uM and node value was assigned as 1 and decay factor was kept as 0.001 to show the longer stay of the drug in the system. Meanwhile ATP was kept at lowest side to see the effect of activator drug on Ca^{2+} response. NF546 induced the Ca^{2+} response was robust and sustained with level of activation maintained at 1.

To further confirm the synergistic effect of ATP and activator drug NF546 on Ca^{2+} signaling both were introduced in the system at highest conc. confirming Ca^{2+} induced signaling due to $P2Y_{11}$ receptor


ATP
Ca2+
GPCR
NF546
ATP, Ca2+ and GPCR response
Time
0
0.2
0.4
0.6
0.8
1
50
100
150
200
250


ATP
Ca2+
GPCR
0
0.2
0.4
0.6
0.8
1
0
100
200
300
400
500


ATP
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GPCR
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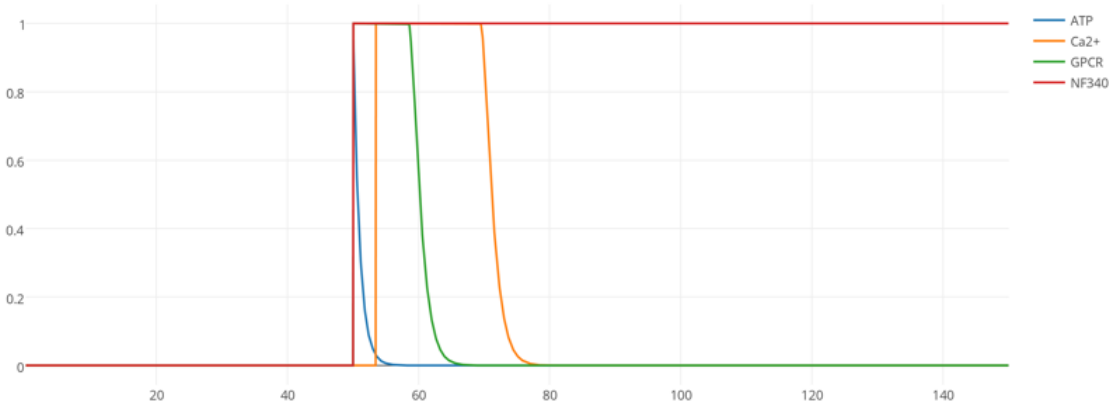
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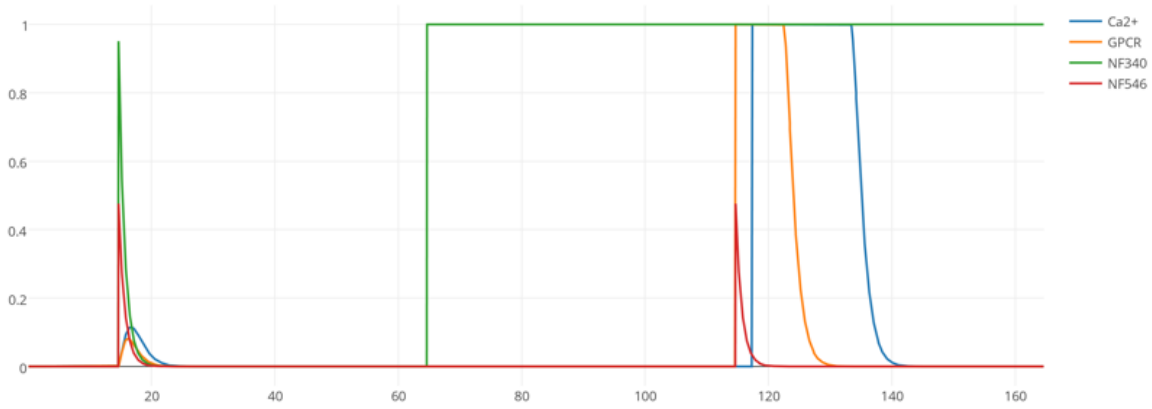

Fig 4.15 Simulation of the effect of activator and inhibitor on receptor activity.

Representative graph were plotted using ploty.ly. Model started at normal state 0, and after a period of time the system is perturbed giving it a saturating pulse signal of ATP, Ca^{2+}, P2Y11, NF546, and NF340. ATP (1µM) induced calcium response b. ATP (100uM) induced calcium response c. ATP (1µM) and NF546 (10µM) NF546 induced Ca^{2+} response d. ATP (100µM) and NF546 (10µM) ATP and NF546 induced Ca^{2+} response e. ATP (100µM and NF340 1µM) NF340 inhibited ATP induced Ca^{2+} response f. NF546 1µM and 10µM NF340 inhibited NF546 induced Ca^{2+} response.

Chapter 5

DISCUSSION

Hepatocellular carcinoma (HCC) is the major cause of liver disease and death rate is high all over the world (Balogh *et al*., 2016). HCV is highly endemic in Pakistan. Around 6.8% of general population in Pakistan is infected with HCV (Umer & Iqbal, 2016). It is well established that liver injury with cellular stress and inflammation is a potent trigger for ATP release from hepatocytes, immune cells and other liver cell types (Dubyak, 2012; Xiao *et al*., 2012). Adenosine 5′-triphosphate (ATP) has been found to be a potent autocrine/paracrine signaling molecule in hepatocytes (Manzoor, *et al*., 2011). When there is release of un-metabolized ATP and nucleotides in liver cells they ultimately bind to purinergic receptors (P2X) and subsequently activate downstream signaling molecules (Fausther *et al*., 2012).

Previously, there have been reports showing different mRNA expression of P2X receptors in human hepatoma cells stably expressed with envelop protein HCV E1E2 (Manzoor *et al*., 2011). There are also findings that show mRNA expression of isoforms of P2X receptor proteins on the PBMCs which is consistent with the finding of many studies (Manzoor *et al*., 2016). P2X receptors are expressed on immune cells and have their role in many cellular functions of PBMCs that include activation of immune cells, release of cytokines, and strengthening of the antigenic signals (Ferrari *et al*., 2006; Ohsawa *et al*., 2007; Lecut *et al*., 2009; Abramowski *et al*., 2014). The conducted study did not solely focus on the prevalence of these receptors on explanted HCC tissues but it also focuses on the targeted receptors investigation to establish its pro- or antiviral role. Total of 50 patients from ex-planted liver tissue samples of HCV HCC and non-HCV HCC were used. Among 28 patients in HCV

HCC, 5/28 (17.8%) showed low expression of P2X4 and 23/28 (82.1%) showed moderate expression of P2X4 in HCV HCC. While among 22 patients in HCV HCC, 15/22 (68%) showed low expression of P2X4 and 7/22 (31%) showed moderate expression of P2X4 in non-HCV HCC. Comparatively, above mentioned statistics indicate a higher expression of P2X4 receptors in HCV HCC. Numerous P2X receptors are expressed on mammalian cells in cell specific manner that play a vital role in various pathophysiological manifestations including cancers. This moderate expression of P2X4 receptor can be correlated with cellular insult owing to virus that may cause surplus release of ATP in the cellular environment (Manzoor et al., 2011).This released ATP can effect cancer cell functions most importantly cancer cell migration and proliferation (Madiha *et al*., 2017). The current study is also correlated with our previous findings of Manzoor *et al* that shows increased levels of numerous variants of P2XR while in the presence of HCV envelop proteins E1E2 (Manzoor *et al*., 2011).

Similarly, when we measured the expression of P2X7 in HCV HCC, Among 28 patients in HCV HCC, 21/28 (75%) showed low expression of P2X7 and 7/28 (25%) showed moderate expression of P2X7 while among 22 patients in HCV HCC, 14/22 (63%) showed low expression of P2X7 and 8/22 (36%) showed moderate expression of P2X7 in non-HCV HCC. We found that P2X7 receptor expression was almost unresponsive in ex-planted liver tissue samples both in non-HCV and HCV HCC. This might give us a clue that P2X7 is most widely expressed on peripheral blood mononuclear cells (PBMCs) and other immune cells that's why play a significant role in inflammatory responses by immune cells. Previous reports have shown that P2X7 receptor expression and activation provoke immune cells to eradicate viral infection. (Ashraf *et al*., 2013). We may also suggest the fact that P2X7

is a regulator of several key inflammatory molecules, including IL-1β, IL-18, TNF-alpha and IL-6 (Taylor & Han, 2010) so it might contribute towards the anti-viral responses during liver damage.

Present study suggests that HCV HCC may have relatively rapid upregulation of purinergic receptors as compared to non-HCV HCC. Furthermore, a rapid increase in the purinergic receptors leading to HCC, may prove a precursor for the cancer diagnostics as well. The difference among the percentage increase in purinergic receptors may facilitate for the analysis of cancer stage.

It has been observed that enveloped viruses infect eukaryotic cells by fusion of the viral and plasma membrane. Specific viral envelop proteins catalyze this reaction for effective interaction. Peptide fusion is the first step towards membrane fusion (Suárez *et al*., 2000). HCV envelop protein E1 between residues 309 and 340, is located in close proximity of the TM domain, found to be conserved in all strains of HCV (Pérez-Berná *et al*., 2008). CD81 is a recognized host receptor for HCV entry (Pileri *et al*., 1998). Other candidate receptors involved in the productive infection are SR-BI, class B, type I scavenger receptor, LDL-R, low density lipoprotein receptor, and the last identified Claudin 1 and Occludin (Evans *et al*., 2007; Lindenbach *et al*., 2006). Numerous variants of P2XRs are present extensively in different organs (Evans *et al*., 2007). In previous studies, P2XR types have been found in rat hepatocytes at transcriptional level (Lindenbach *et al*., 2006).

There are previous reports on the presence of different transcripts of P2X receptors in both Huh-7 and Huh-7 stably transfected with HCV envelop protein E1E2 (Manzoor *et al*., 2011). Current study is an attempt to find out any feasible interactions between P2X4 receptors present in the human liver surface and HCV E1 protein to lay down the basis for the viral exploitation of these receptors to augment

the disease pathology. Available crystal structure of HCV E1 viral entry protein with the generation of suitable model of P2X4 from existing Zebrafish model permit the analysis of protein-protein interaction study with numerous observations of potential residues in CPORT. Ten models were selected on the basis of scores provided by ERRAT, QMEAN and MODELLER. ERRAT scores were in the range of ~50-60. On the basis of highest ERRATs score and lower energy values best model was selected.

PPI were studied by using default Guru interface HAADOCK but results come out to be unusual there were implausible interactions between HCV E1 and human P2X4 receptors. Latter, it was found that removing stretch and strains during loop refinement might hinder interacting residues. So the PPIs were repeated with structure without refinement and astonishingly there were plausible interactions between two proteins further confirmed with Guru Interface protein interactions and structure were validated on HADDOCK refinement interface. PPI studies of P2X4 and E1 revealed that Viral E1 might competitively bind liver cell surface receptors through polar contacts between amino acid residues at a distance of 3.9 $A^{0.}$ We identified amino acid residues in the C-terminal of P2X4 receptor protein were Gly-960 , Gly-641, ASP-974, ASP-648 and aminoacids in the N-terminal of viral E1 were ALA-17, ASN-12, MET-5, ARG-4, HIS-3. Already available Crystal structure of P2X7 (Caseley *et al*., 2015) was auto docked with viral envelop E1 protein. We docked the protein at lowest energy and most populated area and amino acids were identified. Lower energy P2X7 receptor's interactions were ASN-187, Gly-185, ARG-244, PHE-240 with HCV E1 protein residues naming THR-16, ASP-8, ARG-26 respectively. And most populated interaction between P2X7 and E1 protein within 3.9 A^{0} was TYR-317 and ASN-12.

This study also implicates that HCV virus exploits available receptors on the cell surface to worsen the disease pathology. Although these receptors have not been studied until now for the virus entry into the liver cell but they can be exploited for targeting worsening situation in liver pathology.

Activation of GPCRs after binding with ATP leads to downstream activation of calcium and other signaling proteins. Binding of IP3 with Akt moves the cell towards cell survival. Continuous sensitization of GPCR causes intracellular influx of calcium that can keep Akt as activating and suppression of tumor suppressor genes can lead towards cell proliferation and migration in cancer cells. Since calcium levels are high in Huh-7 and HepG2 cell lines when exposed to different ATP concentrations. This high levels of ATP can keep cell in survival state with suppression of tumor suppressor genes as observed in final cycles where receptor is activated and calcium levels are high. This can ultimately lead to deadlock state that is the proliferation of liver cells. To examine the role of $P2Y_{11}$ receptors in ATP-induced increase in the $[Ca^{2+}]_i$, we determined ATP-induced Ca^{2+} response initially at minimum conc. of ATP. In this case node values assigned as 0 for ATP, $P2Y_{11}$ and Ca^{2+} with decay factor of 1 shown in table 1. Both GPCR and Ca^{2+} are show in normal state and ATP is supposed to add at minimal concentration of 1uM in the system to observe the change in Ca^{2+} response. There was an increased response of Ca^{2+} when ATP was added in the system, but it drops down suddenly after the ATP is decayed after 300 sec. to obtain optimum dose response model was given with high con. of ATP (100µM). High conc. was assigned node value of 1. There was a robust response in the Ca^{2+} response that became sustained after some time.

Our next target was to use the activator drug NF546 that can mimic the ATP induced- Ca^{2+} response in the same manner. NF546 was added at the conc. of 10µM

and node value was assigned as 1 and decay factor was kept as 0.001 to show the longer stay of the drug in the system. Meanwhile ATP was kept at lowest side to see the effect of activator drug on Ca^{2+} response. NF546 induced the Ca^{2+} response was robust and sustained with level of activation maintained at 1. To further confirm the synergistic effect of ATP and activator drug NF546 on Ca^{2+} signaling both were introduced in the system at highest conc. confirming Ca^{2+} induced signaling due to $P2Y_{11}$ receptor.

The current study proves that ATP-induced Ca^{2+} signaling confirms the functional expression of $P2Y_{11}$ receptor pharmacologically in Huh-7 cells. This study was strengthened with the finding of $P2Y_{11}$ in human HCC cells genetically and cancer-specificup-regulation of $P2Y_{11}$ receptor in HCC cells (Madiha *et al*., 2017). Firstly, we showed that ATP induced an increase in the $[Ca^{2+}]_i$ in Huh-7 cells. These ATP-induced Ca^{2+} responses were sensitive to inhibition by PPADS and suramin. Collectively, ADP was much less effective than ATP in elevating the $[Ca^{2+}]_i$. These results are consistent with previous findings of Huh-7 cells (Schöfl *et al*., 1999) and also with those in a recent study of native human HCC cells and HepG2 and BEL-7404 cells (Xie *et al*., 2014). We also showed that the $P2Y_1$ receptor agonist MRS2365 was ineffective in inducing an increase in the $[Ca^{2+}]_i$. This has ruled out the major role of the $P2Y_1$ receptor notwithstanding its mRNA expression earlier reported in Huh-7 cells (Schöfl *et al*., 1999). UTP was as potent as ATP in elevating the $[Ca^{2+}]_i$. These results could help find the involvement of the $P2Y_2$ receptor too (Schöfl *et al*., 1999). Certainly, a current study have shown shRNA-mediated knockdown of the $P2Y_2$ receptor reduced the ATP-induced increase in the $[Ca^{2+}]_i$ in HepG2 and BEL-7404 cells, supporting functional expression of the $P2Y_2$ receptors in these cells (Xie *et al*., 2014). $P2Y_2$ receptor agonist MRS2768 failed to induce any

significant increase in the $[Ca^{2+}]_i$ in Huh-7 cells which is not consistent with an important role of the $P2Y_2$ receptor in Huh-7 cells.

We further examined the expression of $_{P2Y11}$ (ATP-sensitive P2Y receptor) receptor which was not investigated in previous studies. $P2Y_{11}$ receptor agonist, NF546 was also equally effective as ATP in provoking robust Ca^{2+} responses in Huh-7 cells. Furthermore, pre-treated huh-7 cells with $P2Y_{11}$ receptor specific antagonist NF340 abrogated ATP-induced Ca^{2+} response. Immunofluorescent confocal images showed Huh-7 cells showed positive $P2Y_{11}$ receptor protein expression. ATP-induced Ca^{2+} responses in the extracellular Ca^{2+}-free solutions were significantly greater than those in the extracellular Ca^{2+}-containing solutions . Potential mechanism involved are the reduction in the ER Ca^{2+} level, due to Ca^{2+} release following activation of the $P2Y_{11}$-$G_{\alpha,q/11}$-PLC-IP_3R signaling pathway, activates the store-operated Ca^{2+} entry. These store-operated Ca^{2+} signaling mechanism has been recently shown to exist in native human HCC cells and HepG2 and BEL-7404 cells (Xie *et al*., 2014). Alternative mechanism is the P2X receptor that can also mediates extracellular Ca^{2+} influx.

Previous reports have shown in rat HCC cells, BzATP induces large fast-desensitizing inward currents using patch-clamp recording and a rapid extracellular Ca^{2+} influx using Ca^{2+} imaging. P2X4 receptor and possibly the P2X7 receptor were thought to mediate such responses (Emmett *et al*., 2008). While suramin and PPADS were effective in inhibiting ATP-induced Ca^{2+} responses. P2X4 receptor selective antagonist 5-BDBD resulted in no inhibition. Present study affirms the protein expression of the P2X7 receptor in Huh-7 cells. However, there was also no Ca^{2+} response to BzATP which is known to activate the P2X7 and other P2X receptors including the P2X4 receptor (North & Surprenant, 2000). αβmeATP also induced no increase in the $[Ca^{2+}]_I$ signifying no expression of functional P2X receptors

containing the P2X1, P2X3 and P2X5 subunit. It is confirmed from the present study that the P2X4 and P2X7 receptors are unlikely to have a significant role in mediating ATP-induced Ca^{2+} responses in Huh-7 cells, thus differing from rat HCC cells whereas further studies are required to examine the potential contribution of P2X2 and P2X6 receptors (Emmett *et al*., 2008). It is evident from the current study that there is no convincing interpretation for such a discrepancy. There may a specie difference so we need to exercise caution in using rodent cells and disease models to elucidate the molecular mechanisms underlying human HCC.

Emerging evidence suggests high micromolar concentrations of extracellular ATP at the tumour sites (Falzoni *et al*., 2013; Pellegatti *et al*., 2008). Here, we showed that ATP was potent in increasing the $[Ca^{2+}]_i$ with an EC_{50} of 11 µM. Thus, use of selective antagonists against P2Y11 suggests a potential role for the $P2Y_{11}$ receptor in ATP-induced stimulation of Huh-7 cell migration. Further preclinical studies can firm a critical role of the $P2Y_{11}$ receptor in ATP-induced regulation of HCC cell migration and metastasis *in vivo*. It is feasible to explore the therapeutic promise of targeting the $P2Y_{11}$ receptor for HCC treatment as a number of $P2Y_{11}$ selective antagonists are already in clinical uses, mainly as anti-platelet drugs (von Kügelgen & Harden, 2011).

In summary, our study provides strong evidence to show that expression of the $P2Y_{11}$ receptor is strongly up-regulated and has a critical role in ATP-induced Ca^{2+}signaling mechanism. Such findings are important for not only understanding of the pathogenesis of HCC but also for identification of disease biomarkers and drug targets in development of new diagnosis and therapeutic approaches to HCC.

To conclude overall story, we report expression of purinoceptor P2X4 expression on ex-planted liver tissue samples. In addition, there were weak protein interactions

between P2X4 and HCV E1. With competitive binding through polar contacts between amino acid residues (C-terminal of P2X4 receptor protein were Gly-960, Gly-641, ASP-974, ASP-648 and amino acids in the N-terminal of viral E1 were ALA-17, ASN-12, MET-5, ARG-4, HIS-3) at a distance of 3.9 $A^{0.}$ Lower energy P2X7 receptor's interactions were ASN-187, Gly-185, ARG-244, PHE-240 with HCV E1 protein residues naming THR-16, ASP-8, ARG-26 respectively. And most populated interaction between P2X7 and E1 protein within 3.9 A^0 was TYR-317 and ASN-12. $P2Y_{11}$ receptor is strongly up-regulated in a human HCC-specific fashion and has a critical role in ATP-induced Ca^{2+} signaling mechanism. Furthermore $_{P2Y11}$ receptor has role in ATP induced liver cell migration. In silico studies confirm mutated GPCR signaling pathway leads to a deadlock state (metastasis) that is a key process in cancers.

CONCLUSION

These findings constitute the evidence that P2X4 receptors expression was significantly increased in HCV HCC as compared to non-HCV HCC. Whereas P2X7 receptor expression was unresponsive in both HCV HCC and non-HCV HCC. This might give us a clue for P2X4 receptor being proviral. To best of our knowledge we support the following findings in current study; Current Study confirmed the presence of P2X4R in Huh7 cell line. P2X4R is functionally active as determined by the agonist (ATP) activity and changes in Ca^{2+} levels measured by F340/F380. Our study provided the human P2X4R model for further studies. PPI studies of P2X4 and E1 revealed that Viral E1 might competitively bind liver cell surface receptors through polar contacts between amino acid residues at a distance of 3.9 $A^{0.}$ We identified amino acid residues in the C-terminal and N-terminal of P2X4 receptor protein were Gly-960 , Gly-641, ASP-974, ASP-648 and ALA-17, ASN-12, MET-5, ARG-4, HIS-3 respectively that are involved in receptor viral interaction facilitating viral entry. Lower energy P2X7 receptor's interactions were ASN-187, Gly-185, ARG-244, PHE-240 with HCV E1 protein residues naming THR-16, ASP-8, ARG-26 respectively. And most populated interaction between P2X7 and E1 protein within 3.9 A^0 was TYR-317 and ASN-12.This study implicates that Hepatitis C virus exploits available receptors on liver cell surface to worsen the disease pathology. Although purinergic receptors have not been studied yet for the virus entry into the liver cells but they can be exploited for targeting worsening disease conditions of liver. Our results show carcinoma-specific expression of the $P2Y_{11}$ receptor and its critical role in mediating

71 inducing Ca^{2+} signaling and regulating cell migration in human HCC cells.

FUTURE PROSPECTS

This study can be further extended to find out the role of $P2Y_{11}$ in HCC. As already established in HCC migration, with the help of viral proteins and animal disease models specific for hepatocellular carcinoma can help find out the specific role of P2 receptors particularly $P2Y_{11}$ in liver cancer. Once the role of P2 receptors is established in hepatocellular carcinoma, further agonist and antagonist studies can facilitate the discovery of suitable drugs. These studies can lead us to find specific therapeutic regimens and best suitable targets for devastating liver cancer in growing world population.

Chapter 6

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APPENDICES

APPENDIX I (SOLUTION / MEDIA COMPOSITION)

Ca^{2+} free Standard Buffer Solution (1 L, omM Ca^{2+})

NaCl	7.85g
KCl	0.37g
Glucose	1.44g
HEPES	2.38g
MgCl2	1.2ml
EGTA	0.15g

Standard Buffer Solution with Ca^{2+} (1L)

NaCl	7.85g
KCl	0.37g
Glucose	1.44g
HEPES	2.38g
$MgCl_2$	1.2ml
$CaCl_2$	1.5ml

- PA in 10% DMSO
- Fura-2 AM 50 µg lyophilized by adding 50 µL of DMSO
- Dulbecco Modified Eagle's Medium (DMEM) supplemented with 100U/ml of penicillin and 100ug/ml of streptomycin and 10% heat inactivated fetal bovine serum

Wavelength	
Excitation	340
Emission	380
Emission cut off	525
sensitivity	
Readings	3
timings	
Time	180 sec
Interval	2-5 sec
Assay Plate type	Greiner 96-well plate
Wells to read	1-12 columns
Compound source	Costar 96 well plate clear flat bottom
Compound transfer	
Transfers	1
Initial Volume	180ul
Pipette Height	110ul
Transfer volume	40ul
Dispense speed	3
Transfer time	30sec
Pipette layout	Depending on experimental design
Compound & tip column	Depending on experimental design

APPENDIX III (PUBLICATIONS)

In the line of research conducted, the following manuscripts have been furnished:

- Madiha Khalid, Sobia Manzoor. Structural evaluation of Purinergic receptor (P2X4) interaction with HCV envelop protein E1: A potential target for Drug. International Conference on Recent Advances in Medical and Health Sciences (ICRAMHS) Rawalpindi, Pakistan 23rd-24th July, 2016, organized by ACADEMICS WORLD and in association with PET for presentation at the Conference.

- Madiha Khalid, Menahil Tariq, YunjieHao, Sharifah Alawieyah Syed Mortadza, FatemaMousawi, Sobia Manzoor and Lin-Hua Jiang. A critical role for the P2Y11 receptor in extracellular ATP-induced purinergic stimulation of human hepatocellular carcinoma cell migration. Oncotarget.2017;8(23):37278-37290).