Pharmacological evaluation of alkyl chain-linked thiourea derivatives as P2Y1 Receptor antagonist

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Abstract- P2Y1Rs (GPCRs) have pathophysiological importance, like immune system regulation, inflammation, blood aggregation and vascular system. Other than these, P2Y1R expressed in many cancers importantly, melanoma and prostate (PC3). In the current study alkyl chain-linked thiourea derivatives were investigated as P2Y1 receptor antagonists. Among the tested compounds, the most potent P2Y1R antagonists showed highest cell viability and they were non-toxic, compound 6q showed 49.66% cell viability in (MTT) assay. The Ca2+ mobilization assay was used to get the most potent antagonists of P2Y1 receptors, compounds 1q, 4q, 5q and 6q showed IC50 values 1.27, 0.133, 0.486 and 0.126 µM, respectively. They were inactive/non-selective against tP2Y1, hP2Y2, hP2Y and rP2Y6 receptors. Most of the compounds showed excellent predicted pharmacokinetic properties performed with SwissADME. The nature of interaction of most potent antagonists (1q, 4q,5q and 6q) of P2Y1 receptors was determine through molecular docking studies. The investigated antagonists showed same binding interactions in the active site of the receptors compared with standard antagonist (BPTU). The obtained results suggested that alkyl chain linked thiourea derivatives were P2Y1 receptor antagonists, the tested compounds could be suitable for anticancer drug development.

Key words: Antagonist Ca^{2+} assay, *in silico* studies, P2Y1R

ABBREVIATIONS

ADP; adenosine diphosphate

ATP; adenosine triphosphate

BPTU; 1-(2-(2-(tert-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)phenyl)urea

ERK1/2; extracellular signal-regulated protein kinase 1/2

IP3; inositol triphosphate

MTT; a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MAPK; mitogen-activated protein kinase

PLC; phospholipase C

TPSA; polar surface area

1. INTRODUCTION

The P2Y receptors are G protein-coupled receptors (GPCRs), they consist of eight subtypes: the five of them are Gq/G11-coupled subtypes (P2Y1R, P2Y2R, P2Y4R, P2Y6R and P2Y11R)[1]. Usually activating phospholipase C and IP3 production leads to endoplasmic reticulum calcium release, and three Gi/o-coupled subtypes (P2Y12, P2Y13 and P2Y14), mainly inhibiting adenylyl cyclase [2]. P2Y1R receptor is one of the P2Y receptors family member whose activation by ADP is related to the Gq signaling cascade. PLCβ activation further produce IP3 and DAG generation from membrane phospholipids which further leads, increased Ca2+ release from endoplasmic reticulum and PKC activation[3]. Pathological role of P2Y1R expression associated with different disease conditions most importantly in several cancers[4]. In PC-3 prostate cancer cells, the Gq-coupled P2Y1 nucleotide receptor is expressed at high levels[5].

P2Y1 receptors are prominently expressed in vascular tissues such as smooth muscle cells, platelets, and endothelial cells, where they regulate vascular tone and blood pressure. Activation of P2Y1 receptors by ADP or ATP in vascular smooth muscle cells leads to an increase in intracellular calcium concentrations, resulting in vasoconstriction [6]. This process plays a crucial role in physiologic blood pressure regulation and maintaining appropriate blood flow to different organs. In addition to their role in vasoconstriction, P2Y1 receptors have been implicated in regulating platelet activation. Moreover, P2Y1 receptors have been shown to take part in immune system regulation and the modulation of inflammation[7]. P2Y1R activation in cancer cells is often associated with anti-proliferative response and may contribute to cancer cell metastasis[8]. P2Y1 and P2Y6 receptors were found in abundance and uniform expression in human melanoma tissue, but P2Y2 receptors were found primarily at the tumor's proliferative boundaries[9]. P2Y1 receptors and their signaling pathway have been demonstrated to play important roles in the progression of various types of cancer, including prostate cancer, ovarian cancer, and others. One study found high gene expression levels of P2Y1 nucleotide receptors in PC-3 prostate cancer cells, leading to the activation of downstream signaling pathways and promoting apoptosis and cell death [10]. Furthermore, the P2Y1R-selective agonist 2-MeS-ADP decreased proliferation in the human melanoma cell line A-375, whereas the P2Y2R-selective antagonist, indicating contrasting roles for these two P2YR subtypes in cancer [11]. A number of cell signaling pathways, such as the protein kinase C pathway, which in turn promotes the mitogen-activated protein kinase (MAPK)-signaling pathway, can connect proteins to P2Y1R. Notably, this mechanism involves the extracellular signal-regulated protein kinase 1/2 (p-ERK1/2) [12]. The in vitro, cancer cell growth is aided by the activation of the ERK and p38 (another member of the MAPK family) signalling pathways[8]. P2Y1 purinoceptor inhibition reduces extracellular signal-regulated protein kinase 1/2 phosphorylation in spinal cord and dorsal root ganglia: implications for cancer-induced bone pain[13]. P2Y1R Pharmacological agonists similar in potency to ADP, 2-Methylthio-ATP and ATP-S similarly operate as agonists at the P2Y1 receptor[14]. Higher potency analogues exist for the human P2Y1 receptor. Included in this is the analogue 2-methylthio-ADP[15]. In comparison to the P2Y12 and P2Y13 receptors, the Northern (N) -methanocarba analogue of 2-methylthio-ADP (MRS2365) is particularly potent[16]. There are several known antagonists of P2Y1R receptors included, affinity antagonists include suramin, PPADS, reactive blue-2, and MRS2210 (6-(2′-chloro-azophenyl)-pyridoxal-5-phosphate)[17]. The P2Y1 receptor has much higher affinities for the bisphosphate analogues MRS2179, MRS2279, and MRS2500 than it does for, say, suramin[18]. On the basis of literature review currently available standard antagonist have low affinity and most of them are non-selective for P2Y1 receptor. In our study we have aimed to find the selective and potent P2Y1 antagonist. Through

random screening a series of published thiourea derivatives[19] were evaluated against P2Y1 receptor to find most potent antagonist. Ca2+ mobilization assay was used in t-P2Y1, h-P2Y2, h-P2Y4, and r-P2Y6-1321N1 astrocytoma cell lines, and fluorescent Ca2+ influx was measured using a Ca2+ binding dye (Fura-2 AM). A cell viability assay (MTT assay) was performed to determined safety profile of compounds. In silico investigations were carried out to confirm the in silico interaction between target and ligand.

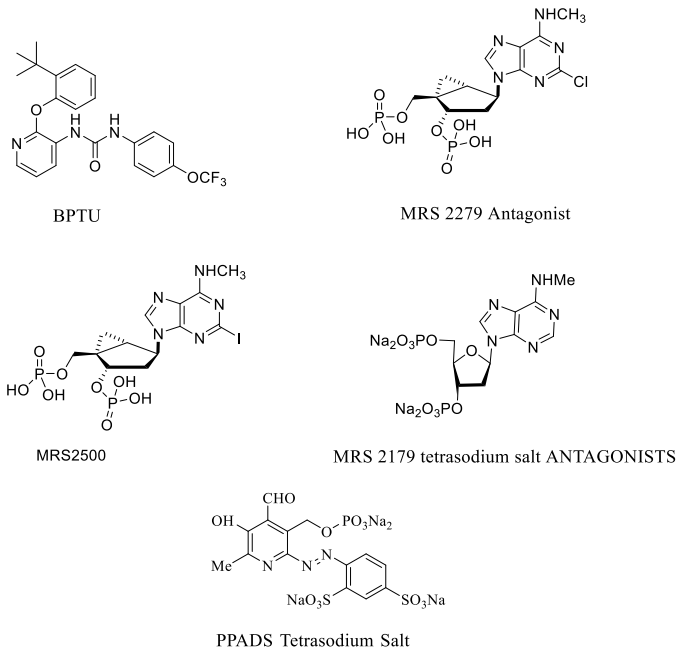


Figure.1: Reported antagonist of P2Y1 receptor

2. RESULTS AND DISCUSSION

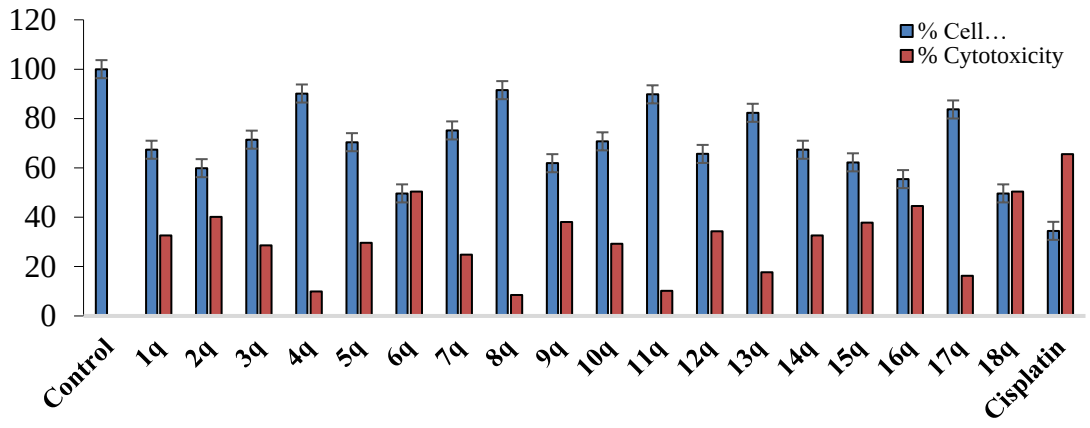


Figure.1: Cell viability of compounds **1q-18q** were evaluated against HEK-293T cells, 100 µM at final concentration. Cell viability was determined after 24-48 hrs. incubation compared to Cisplatin (100 µM). untreated control, DMSO was used. Compounds were tested in triplicate.

Table.1: Cells Viability (%) and Cytotoxicity (%) of synthesized compounds normal HEK-293 T cell lines

Codes	% Cell Viability (HEK-293T)	% Cytotoxicity
Control	100	0
1q	67.35±0.71	32.65±0.42
2q	59.88±0.62	40.12±0.52
3q	71.43±0.86	28.57±0.31
4q	90.14±1.21	9.86±1.20
5q	70.41±0.622	29.59±0.32
6q	49.66±0.32	50.34±0.62
7q	75.17±0.65	24.83±0.34
8q	91.50±0.87	8.50±0.91
9q	61.89±0.71	38.11±0.51
10q	70.75±0.83	29.25±0.33
11q	89.80±0.92	10.20±0.12
12q	65.65±0.75	34.35±0.42
13q	82.31±0.93	17.69±0.19
14q	67.35±0.78	32.65±0.37
15q	62.24±0.68	37.76±0.39
16q	55.44±0.65	44.56±0.45
17q	83.67±0.92	16.33±0.18
18q	49.66±0.55	50.34±0.62
Cisplatin	34.49±0.47	65.51±0.66

*All experiments repeated 3-4 times

2.1 EVALUATION OF COMPOUNDS CELL VIABILITY/TOXICITY

Compounds 1q-18q were tested for their safety profiling by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) cell viability assay. Cisplatin was used as a standard cytotoxic agent in Table 1.

None of tested compound highly toxic for normal HEK-293 cell lines. Highest cells viability has been observed with (4q); 90.1 %, (6q); 82.66 % while minimum cell viability was noted with (2q); 59.88% rest of antagonists were around 70% cell viability. These results (Table.1.) confirmed that all antagonists are eligible for assays having cells are used most importantly Ca^{2+} mobilization assay.

Table.2: IC_{50} values of the synthesized series, as measured in Ca^{2+} mobilization assay against P2Y1 and %age fluorescence at hP2Y2, hP2Y4 and rP2Y6

Compound	R	tP2Y1	hP2Y2	hP2Y4	rP2Y6
		IC_{50}±SEM (μM) [a] (or % Fluorescence ±SD at indicated concentration) b			
1q	2-CH_3	1.27± 0.131	66.2± 0.7%	90.5± 0.1%	88.8± 0.4%
2q	2,3-CH_3	0.158± 0.035	82.3± 0.2%	79.3± 0.5%	89.3± 0.5%
3q	4-OCH_3	1.987± 0.171	77.8± 0.8%	78.5± 0.6%	90.4± 0.3%
4q	2,5-CH_3	0.133± 0.162	83.7± 0.4%	84.7± 0.4%	71.1± 0.2%
5q	4-CF_3	0.486± 0.034	82.5± 0.6%	83.2± 0.6%	93.5± 0.5%
6q	2-NO_2-4-$0CH_3$	0.126± 0.036	93.0± 0.3%	94.1± 0.7%	87.4± 0.2%
7q	4-C_2H_5	15.73± 1.129	87.8± 0.2%	90.5± 0.4%	87.1± 0.1%
8q	4-Br	88.6± 0.4%	68.6± 0.4%	92.7± 0.6%	72.4± 0.7%
9q	4-CF_3	84.5± 0.6%	74.5± 0.6%	83.2± 0.2%	81.6± 0.5%
10q	3,4-Cl	66.6± 0.7%	76.6± 0.7%	79.4± 0.3%	71.7± 0.4%

11q	-Ph	80.9± 0.3%	70.9± 0.3%	93.8± 0.7%	89.6± 0.6%
12q	4-$COCH_3$	90.9± 0.2%	80.9± 0.2%	78.9± 0.4%	85.6± 0.3%
13q	2-SH	80.1± 0.7%	80.1± 0.7%	84.3± 0.8%	79.6± 0.9%
14q	2-OH	89.3± 0.6%	79.3± 0.6%	82.8± 0.3%	88.1± 0.5%
15q	4-CH_3	89.2± 0.8%	79.2± 0.8%	37.2± 0.4%	81.4± 0.3%
16q	4-$N0_2$	70.7± 0.5%	80.7± 0.5%	85.5± 0.6%	93.2± 0.6%
17q	4-NO_2	75.8± 0.6%	85.8± 0.6%	74.4± 0.2%	79.3± 0.7%
18q	2-NO_2-4-OCH_3	80.2 ± 0.3%	70.2 ± 0.3%	86.8± 0.9%	84.8± 0.4%
Suramin		0.19 ± 0.01	3.31 ±0.08	n.d	n.d

2.2 Ca^{2+} MOBILIZATION DETERMINATION

Ca^{2+} sensitive Fura-2 dye was applied as it chelates with intracellular Ca^{2+} ion released from intracellular endoplasmic reticulum by activation of agonist, in a Gq-protein coupled (P2YRs) receptors. ADP induced Ca^{2+} mobilization assay was performed to determine (P2Y1Rs) antagonistic potential of test compounds. Through this assay some compounds showed good affinity and potency in (μM.) toward P2Y1R. The IC50 values of most potent are (6q); 0.126, (4q); 0.133, (2q); 0.158, (5q); 0.486, (1q);1.27, and (3q); 1.987 μM compared to suramin standard antagonist. Only compound (7q); showed least affinity with IC50 value; 15.73 μM. These results further confirm that P2Y1R antagonists were selective towards only P2Y1R, they did not show any affinity against the tP2Y1, hP2Y2, hP2Y4 and rP2Y6. They showed similar fluorescence to control ADP.

3. COMPUTATIONAL STUDIES FOR P2Y1 RECEPTORS

3.1 MOLECULAR DOCKING STUDIES

In silico docking studies were conducted to explore the manner in which P2Y1 receptor binds with the active site. The crystal structure of P2Y1(PDB ID = 4XNV) was downloaded From protein data bank https://www.rcsb.org/

to recognize the latent binding interactions. Synthesized derivative 65, 87, 115,121and 127 showed the excellent estimated binding affinity 0.40, 0.33, 0.23 and 0.50 in micro molar range towards P2Y1R.

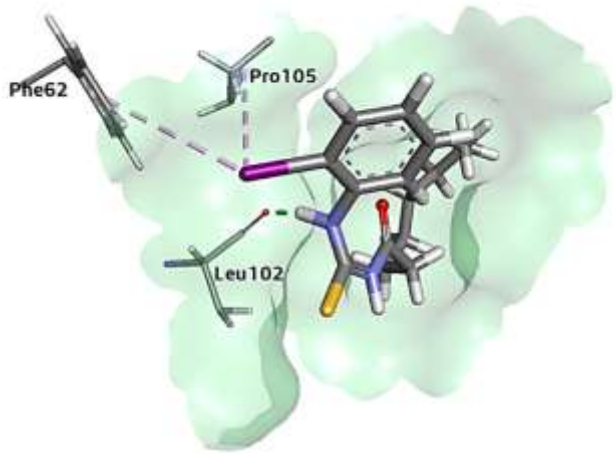
The reported antagonist of P2Y1 receptor BPTU was re-docked in allosteric binding pocket of P2Y1R.Leu102 showed the most promising H-bonding interactions with the nitrogen atoms of the urea linkage. Pi-Pi stacking was observed with amino acids PHE62 and PHE119.Amino acid residue Pro105 made π-Alkyl linkage with phenyl ring while Ala106 and Met123 showed pi-sigma interactions. These mentioned hydrophilic and hydrophobic interactions were considerable for the inhibitory activity of BPTU.

Docking results of **1q** with P2Y1 showed Hydrogen bond and Pi-Alkyl interactions. Hydrogen bond was observed between hydrogen atom of –NH group attached directly with phenyl ring and amino acid LEU102.Iodine atom at ortho position contributed in the formation of π -Alkyl interactions with amino acid residues PHE62 and PRO105.

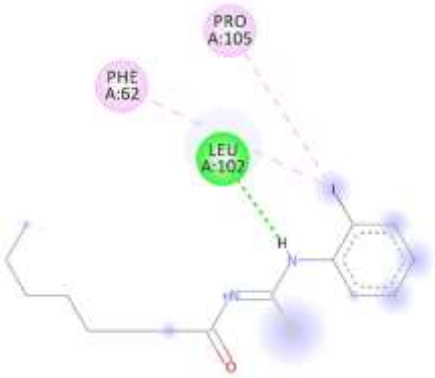
Over all two hydrogen bonds were formed during the docking interaction analysis of **2q** and P2Y1.Hydrogen atom of –NH developed Hydrogen bond with amino acid residue LEU102 While another hydrogen bond was observed between oxygen atom of Nitro substituent and ALA106. Terminal methyl group made π -Alkyl interactions with amino acids ALA106 and MET123.

Docking analysis of 3q showed that Amino acid LEU102 was involved in the formation of Hydrogen bond with Hydrogen atom of –NH group and π -Alkyl interaction with the phenyl ring of compound under study. Methyl group of alkyl side chain developed alkyl interaction with amino acid MET123 π-π stacking was found between phenyl ring and amino acid PHE66.

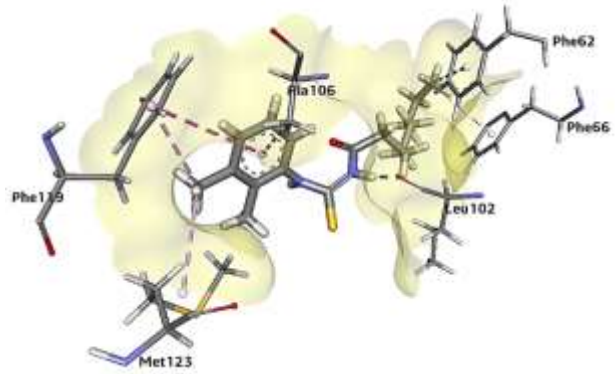
In case of **5q,** Hydrogen bonding was observed between Hydrogen atom of –NH group and LEU102.Same amino acid residue was involved in the development of π -Alkyl interactions with phenyl ring and alkyl side chain of ligand molecule. Two Pi-alkyl interactions between tri-fluoro group and PHE66 as well as phenyl ring and amino acid PRO105 was observed. Pi- π stacking interaction was formed between phenyl ring and amino acid PHE62.
Binding interactions study of **6q** revealed that Hydrogen atom of –NH group made hydrogen bond with LEU102. Another hydrogen bond was found between oxygen atom of Nitro group and ALA106. Amino acid ALA106 made π -Alkyl interaction with alkyl side chain. One Pi-Alkyl interaction was observed between alkyl side chain and amino acid residue MET123.
Study of binding interaction analysis confirms the similar binding patterns of synthesized compounds and standard inhibitor. Probable inhibition potential can be justified from the results of molecular docking studies.


Phe62
Pro105
Leu102

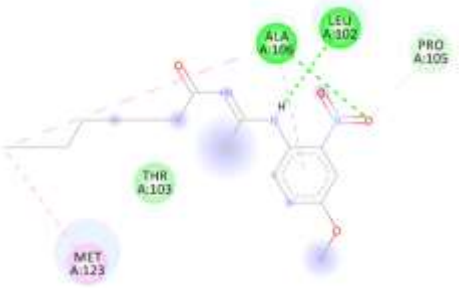
A


PRO
A:105
PHE
A:62
LEU
A:102

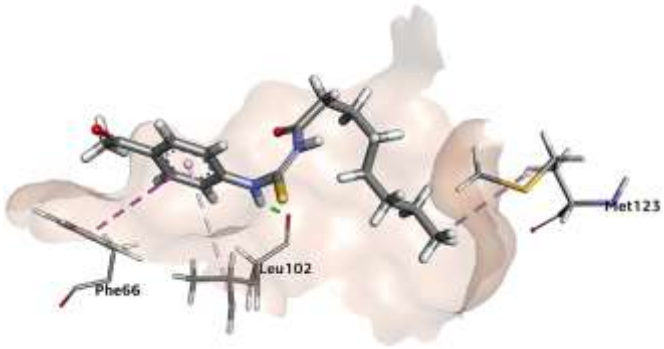
B


Phe62
Ala106
Phe66
Leu102
Met123

C


LEU
A:102
ALA
A:106
PRO
A:105
THR
A:103
MET
A:123

D


Met123
Leu102
Phe66

E


MET
A:123

F

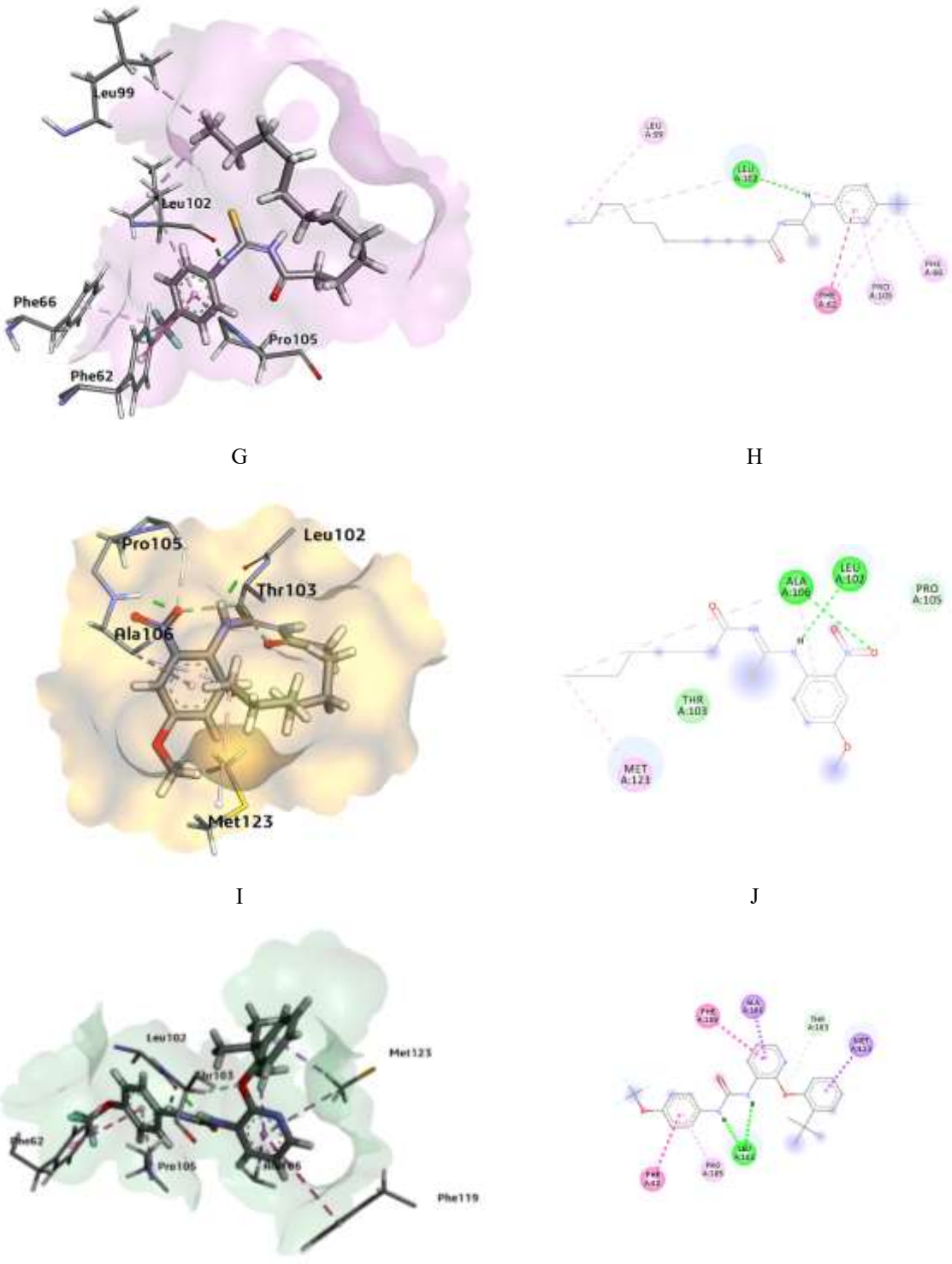


Figure.2: 2D and 3D interaction of the most potent inhibitors Fig.1q,2q,3q,4q,5q and 6q(A-J) and Fig. K and L are the interaction of the Standard BPTU at the active site of the P2Y1 Receptor

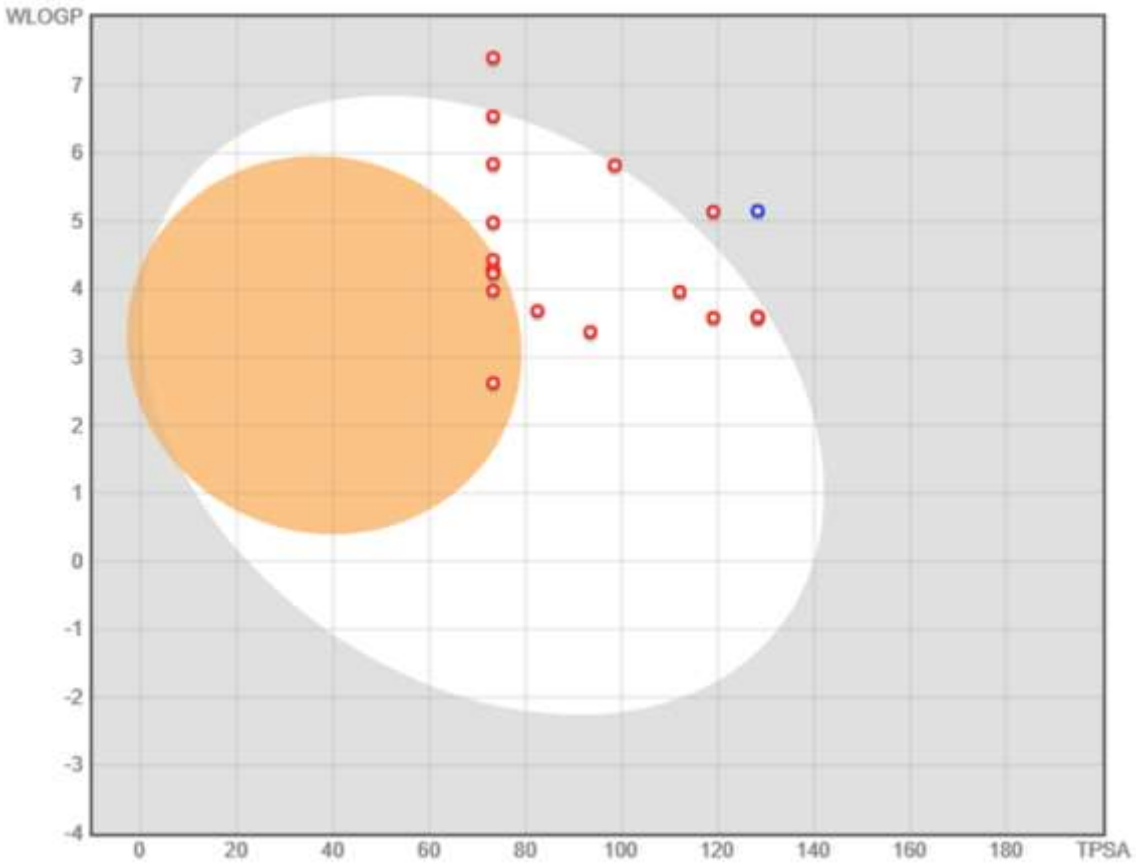


Figure.3:*In silico* predicted ADME boiled egg plot

Table.3:*In silico* evaluation of the synthesized compounds against P2Y1 receptor

Compound Code	MW	#Rotatable bonds	#H-bond acceptors	#H-bond donors	TPSA	iLOGP	WLOGP	GI absorption	BBB permeant	Lipinski #violations	PAINS #alerts
1q	404.31	10	1	2	73.22	3.41	4.27	High	Yes	0	0
2q	306.47	10	1	2	73.22	3.42	4.29	High	Yes	0	0
3q	308.44	11	2	2	82.45	3.23	3.68	High	No	0	0
4q	306.47	10	1	2	73.22	3.38	4.29	High	Yes	0	0
5q	346.41	11	4	2	73.22	3.22	5.84	High	No	0	0
6q	353.44	12	4	2	128.27	3.02	3.59	High	No	0	0
7q	306.47	11	1	2	73.22	3.43	4.23	High	Yes	0	0
8q	357.31	10	1	2	73.22	3.29	4.43	High	Yes	0	0
9q	402.52	15	4	2	73.22	3.96	7.4	Low	No	1	0
10q	403.41	14	1	2	73.22	4.17	6.54	High	No	1	0
11q	353.44	12	4	2	128.27	3.3	3.59	High	No	0	0
12q	256.32	5	1	2	73.22	2.57	2.62	High	Yes	0	0
13q	310.48	10	1	2	112.02	3.09	3.96	High	No	0	0
14q	294.41	10	2	3	93.45	2.23	3.37	High	No	0	0
15q	292.44	10	1	2	73.22	3.18	3.98	High	Yes	0	0
16q	323.41	11	3	2	119.04	2.52	3.58	High	No	0	0
17q	379.52	15	3	2	119.04	3.57	5.14	Low	No	0	0
18q	409.54	16	4	2	128.27	4.07	5.15	Low	No	0	0

P2Y1 antagonist showing in vitro inhibition, many get rejected during clinical trials due to poor physiochemical and pharmacokinetic profiles. Therefore, predication of physicochemical parameters and pharmacokinetic studies at earlier stages to find the suitability for the drug development to reduce cost, resources and labor. The ADME studies of compounds **(1q-18q)** were conducted through *in-silico* based methods (SwissADME)[1] that predicted the parameters such as absorption, distribution, metabolism and excretion of compounds, and the obtained results are mentioned in Table 2 gave important information about the synthetic compounds. The boiled egg plot (generated by Swiss ADME web server) gave overview about the gastrointestinal absorption of all synthetic compounds. The plot, consisting of two parts, the white portion of the egg represents gastrointestinal absorption, and the inner yellow portion represents blood-brain barrier permeability(BBB). While visualizing the boiled-egg plot. All synthesized compounds **1q-18q** display favorable gastrointestinal absorption, and none demonstrated access through the blood brain barrier. All synthesized compounds conformed Lipinski rule of drug absorption, and no violation of this rule was seen[2]. Moreover, drug ability of the compounds was further evaluated through applying PAINS (Pan-assay interference compound) filter[3]. Results suggested that all newly synthesized compounds did not exhibit similarity to PAINS and bear unique structures. All compounds **1q-18q** showed maximum polar surface area (TPSA) between 128.27 and 73.22, and displayed high gastrointestinal absorption. So, after discussing all these parameters, these compounds can be considered as oral drug candidates for further optimization

4. MATERIAL AND METHODS

4.1 CELL CULTURE

Cells were grown in complete culture media Dulbecco's Modified Eagle's Medium (Gibco) addition with 10-15% of (FBS) fetal bovine serum, 1% penicillin, 100 units per mL 100 µg per mL streptomycin and 500 µg/mL and for (P2YRs) transfected media antibiotic G418 was added. Normal non-transfected, HEK-293T and stably transfected 1321N1 astrocytoma cells with (P2YRs) receptors cells were gifted by Dr. Gary A. Weisman (University of Missouri, Bond Life Science Center). Cells were grown in 5% CO2 at 37°C incubator. Cells were used for experiment after reaching at 70-80% confluency.

4.2 CELL VIABILITY/CYTOTOXICITY ASSAY (MTT)

To determine safety profiling of the most potent compounds a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed. Normal cells HEK-293T were grown in 96 well plate (10,00 cells per well) and left for 24 hours in 5% CO2 incubator at 37°C. Test compounds were treated with 100 µM concentration in 96 well plate in triplicate and left some wells as negative control. The cells were further incubated for next 24 hours [20]. MTT reagent was added at concentration of 0.2mg/mL in PBS waited till crystals formation for 2-3 hours at 37°C. The prepared crystals were dissolved by adding pure 100% (DMSO). Absorbance was taken with FLUOstar Omega reader (BMG Labtech, Offenburg, Germany) at wavelengths of 590 and 630 nm. Cell viability was calculated using formula for control: mean OD control / mean OD control *100 =100 %

For test compounds: % Viability = mean OD compound / mean OD control *100

% Cytotoxicity = 100-cell viability

4.3 Ca^{+2} MOBILIZATION ASSAY

P2YRs receptors were expressed in 13121N1 astrocytoma cell lines were grown till 65-85% confluence in 96 well clear bottom black wall plate (40,000 cells per well). Medium was aspirated, cells were washed with HBSS solution. Dye was (Fura-2 AM dye) prepared in HBSS added probenecid at final concentration of 1-3 µM. Incubation was given in the dark at 37°C, for 40-45 minutes. The dye was removed by washing cells with (HBSS) buffer. Different test compounds dilutions were prepared in HBSS buffer and further incubated for half hour at 37 ºC. Agonist ADP and ATP were prepared and injected at a fixed concentration through machine. Dual excitation/emission at 340/520 and 380/520 nm was measured automatically by spectrophotometer. For Ca2+ flux/mobilization assay FLUOstar Galaxy plate reader (BMG Lab Technologies). The plate reader was installed with injectors [21]. For initial screening (percent inhibition) calculated with SEM and for dose response curve and (IC50) Graph Pad Prism 5.0 (Inc., San Diego, CA, USA) was used.

4.3. DOCKING

Docking studies were conducted to understand the binding mechanism of the synthesized thiourea derivatives at the active pocket of the P2Y1 receptor. Molecular docking was performed using the (P2Y1) crystal structure with PDB ID = 4XNV [4]. The targeted proteins was prepared using See SAR (Bio Solve IT GmbH, Germany) [5] and default parameters while the ligands were prepared using Chem3D 15.0 in which the energy minimization of the ligand were done with RMS Gradient 0.0100. The docking studies of the selected and potent inhibitor, poses with the lowest binding energy were examined using Discovery Studio Visualizer DS (Biovia, D. S. 2017) [6].

4.4. SWISS ADME

Physicochemical properties and pharmacokinetic profiles of compounds 1q-18q were determined using the SwissADME http://www.swissadme.ch/ web server. In order to calculate pharmacokinetic profiles of synthesized compounds, chemicals structures were converted to SMILES format using Chemdraw professional (2018) and uploaded to web server to obtained the pharmacokinetics results of the compounds.

5. CONCLUSION

The antagonistic potential of alkyl chain-linked thiourea derivatives (1q-18q) was established against P2Y1 receptors through calcium mobilization assay. Among them compound (1q, 4q,5q and 6q) were most promising with better IC50 values; 1.27, 0.133, 0.486 and 0.126 µM respectively. They were found selective against P2Y1 and were not active at other P2Y receptors. All the antagonists were safe at highest (100 µM) concentration. In silico studies of the most potent antagonists showed similar binding interaction as standard antagonist. Pharmacokinetic profile of all screened compounds exhibited good drugable properties. All the compounds follow Lipinski five rules.

DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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