Uridine Triphosphate Thio Analogues Inhibit Platelet P2Y\textsubscript{12} Receptor and Aggregation

Dursun Gündüz \textsuperscript{1}, Christian Tanislav \textsuperscript{2}, Daniel Sedding \textsuperscript{3}, Mariana Parahuleva \textsuperscript{4}, Sentot Santoso \textsuperscript{5}, Christian Troidl \textsuperscript{1}, Christian W. Hamm \textsuperscript{1} and Muhammad Aslam \textsuperscript{1,*}

\textsuperscript{1} Department of Cardiology/Angiology, University Hospital Giessen, 35392 Giessen, Germany; dursun.guenduez@med.uni-giessen.de (D.G.); christian.troidl@med.uni-giessen.de (C.T.); christian.hamm@med.uni-giessen.de (C.W.H.)

\textsuperscript{2} Department of Neurology, University Hospital Giessen, 35392 Giessen, Germany; Christian.Tanislav@neuro.med.uni-giessen.de

\textsuperscript{3} Department of Cardiology/Angiology, Hannover Medical School, 30625 Hannover, Germany; sedding.daniel@mh-hannover.de

\textsuperscript{4} Department of Cardiology/Angiology, University Hospital Marburg, 35043 Marburg, Germany; mariana.parahuleva@innere.med.uni-giessen.de

\textsuperscript{5} Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, 35392 Giessen, Germany; sentot.santoso@med.uni-giessen.de

* Correspondence: muhammad.aslam@med.uni-giessen.de; Tel.: +49-641-99-42248; Fax: +49-641-99-42259

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Abstract: Platelet P2Y\textsubscript{12} is an important adenosine diphosphate (ADP) receptor that is involved in agonist-induced platelet aggregation and is a valuable target for the development of anti-platelet drugs. Here we characterise the effects of thio analogues of uridine triphosphate (UTP) on ADP-induced platelet aggregation. Using human platelet-rich plasma, we demonstrate that UTP inhibits P2Y\textsubscript{12} but not P2Y\textsubscript{1} receptors and antagonises 10 \(\mu\)M ADP-induced platelet aggregation in a concentration-dependent manner with an IC\textsubscript{50} value of \(~250\) \(\mu\)M. An eight-fold higher platelet inhibitory activity was observed with a 2-thio analogue of UTP (2\(\text{S}\)S-UTP), with an IC\textsubscript{50} of 30 \(\mu\)M. The 4-thio analogue (4\(\text{S}\)S-UTP) with an IC\textsubscript{50} of 7.5 \(\mu\)M was 33-fold more effective. A three-fold decrease in inhibitory activity, however, was observed by introducing an isobutyl group at the 4\(\text{S}\)S-position. A complete loss of inhibition was observed with thio-modification of the \(\gamma\) phosphate of the sugar moiety, which yields an enzymatically stable analogue. The interaction of UTP analogues with P2Y\textsubscript{12} receptor was verified by P2Y\textsubscript{12} receptor binding and cyclic AMP (cAMP) assays. These novel data demonstrate for the first time that 2- and 4-thio analogues of UTP are potent P2Y\textsubscript{12} receptor antagonists that may be useful for therapeutic intervention.

Keywords: 2\(\text{S}\)-UTP; 4\(\text{S}\)-UTP; P2Y\textsubscript{12} receptor; ADP; platelet aggregation

1. Introduction

Platelets play a central role in vascular haemostasis. Uncontrolled platelet activation under certain pathological conditions may result in thrombus formation and occlusion of the vessels, leading to life-threatening cardiovascular events such as myocardial infarction [1,2] and thrombosis [3,4]. Platelets express nucleotide receptors P2Y\textsubscript{1} and P2Y\textsubscript{12} that are activated by adenosine diphosphate (ADP) and play a central role in platelet activation and aggregation [5–8]. Both P2Y\textsubscript{1} and P2Y\textsubscript{12} receptors are G-protein-coupled receptors that are coupled to G\textsubscript{q} and G\textsubscript{i}, respectively [7]. P2Y\textsubscript{1} receptor activation triggers Ca\textsuperscript{2+} mobilisation from the platelet dense tubular system and shape change [9,10], whereas activation of P2Y\textsubscript{12} receptor causes an inhibition of adenyl cyclase-dependent cyclic AMP (cAMP) production and platelet aggregation [7,11]. Because of its central role in thrombus formation and
stabilisation, the P2Y\textsubscript{12} receptor is a well-established target for anti-thrombotic drug development [12]. Presently, only a few P2Y\textsubscript{12} receptor antagonists (both reversible and irreversible) are available for clinical interventions, and because of their pharmacokinetic profiles they have limitations in clinical application [12]. Therefore, there is a need for the development of novel P2Y\textsubscript{12} receptor antagonists. Currently used P2Y\textsubscript{12} receptor antagonists such as clopidogrel are either pro-drugs, requiring conversion to active metabolites by liver metabolic machinery, or are different nucleotide derivatives such as ticagrelor that reversibly bind to P2Y\textsubscript{12} receptor and thus inhibit its activation. Uridine triphosphate (UTP) [13,14] and its thio-derivatives [15] act as natural and synthetic ligands for purinergic P2Y\textsubscript{2} and P2Y\textsubscript{4} receptors, respectively. The present study analysed the anti-platelet aggregation activity of UTP and a number of different thio-analogues, including UTP\textsubscript{\gamma}S, aminoallyl UTP (AA-UTP), 2-thio UTP (2\textsubscript{S}-UTP), 4-thio UTP (4\textsubscript{S}-UTP), and 4-thio-isobutyl UTP (4\textsubscript{S}-ib-UTP). The study was carried out on platelet-rich plasma (PRP) isolated from freshly obtained blood from healthy human volunteers.

2. Results

2.1. Uridine Triphosphate (UTP) Thio-Analogues Antagonise Adenosine Diphosphate (ADP)-Induced Platelet Aggregation with Various Potencies

Figure 1 shows the structure of UTP and its analogues that were tested for anti-platelet aggregation activity in the present study. ADP at concentration of 10 \(\mu\)M caused 75\%–90\% platelet aggregation as measured by change in the turbidity of PRP (Figure 2). UTP antagonised ADP-induced platelet aggregation weakly with an IC\textsubscript{50} value of \(~250\) \(\mu\)M (Table 1). The 2-thio derivative of UTP (2\textsubscript{S}-UTP; compound 2) displayed a stronger inhibition of platelet aggregation (Figure 2A) with an IC\textsubscript{50} of \(~30\) \(\mu\)M (Table 1). There was a 33-fold increase (vs. UTP) in platelet inhibition by thio (–\textsubscript{S}–) modification of –\textsubscript{O}– at position 4 of the pyrimidine ring. This compound (4\textsubscript{S}-UTP) was able to inhibit ADP-induced platelet aggregation completely at 15 \(\mu\)M (Figure 2B) with an IC\textsubscript{50} of \(~7.5\) \(\mu\)M. Addition of an isobutyl group at the 4\textsubscript{S} position to yield 4\textsubscript{S}-ib-UTP, however, resulted in a three-fold decrease in inhibitory activity (Figure 2C). The non-hydrolysable stable analogue of UTP, UTP\textsubscript{\gamma}S, showed very little activity against ADP-induced platelet aggregation (Figure 2D). Likewise, the aminoallyl derivative of UTP (AA-UTP) was virtually devoid of any anti-platelet activity. The IC\textsubscript{50} values of these agents calculated from platelet aggregation data are given in Table 1. In addition to ADP, other agonists such as collagen cause the release of ADP from platelet dense granules thus indirectly activating P2Y\textsubscript{12} receptor which is also involved in collagen-induced platelet aggregation. Therefore, it was investigated whether UTP analogues could also antagonise collagen-induced platelet aggregation. In this context experiments were performed only with the most active agent, 4\textsubscript{S}-UTP. Indeed 4\textsubscript{S}-UTP was able to strongly antagonise the collagen-induce platelet aggregation (Figure 2E).

Table 1. IC\textsubscript{50} values of UTP analogues.

| Compound Number | Ligand       | IC\textsubscript{50} (\(\mu\)M) |
|-----------------|--------------|-------------------------------|
| 1               | UTP          | 250                           |
| 2               | 2\textsubscript{S}-UTP | 30                             |
| 3               | 4\textsubscript{S}-UTP | 7.5                            |
| 4               | 4\textsubscript{S}-ib-UTP | 23                             |
| 5               | UTP\textsubscript{\gamma}S | \(>1000\)                      |
| 6               | AA-UTP       | \(>1000\)                     |
Figure 1. Structure of the compounds used in the study. The marked site shows the modification of the parent compound uridine triphosphate (UTP). Red circle mark the modified position in the original compound 1.
Figure 2. UTP thio-analogues antagonise adenosine diphosphate (ADP)-induced platelet aggregation. Representative tracing of platelet aggregation induced by ADP (10 µM) in the absence or presence of: 2S-UTP (A); 4S-UTP (B); 4S-isobutyl UTP (C); and UTPγS (D). Representative tracings from four experiments using independent PRP preparations. (E) Representative tracing of platelet aggregation induced by collagen (2 µg/mL) in the absence or presence of 4S-UTP (25 µM).

2.2. Binding of UTP Thio-Analogues to P2Y12 Receptor, Cyclic AMP (cAMP) Production, and Vasodilator-Stimulated Phosphoprotein (VASP) Phosphorylation

ADP-induced platelet aggregation is mainly dependent on activation of platelet P2Y12 receptor. In order to confirm that UTP thio-analogue-mediated inhibition of ADP-induced platelet aggregation is due to antagonism at P2Y12 receptor, radioligand-displacement assays were performed using [3H]-ADP as the P2Y12 agonist in the presence of P2Y1 antagonist (MRS2500 10 µM). As shown in Figure 3A, saturating concentrations of UTP thio-analogues competitively antagonised the binding of [3H]-ADP to P2Y12 receptor. 4S-UTP showed the strongest displacement of [3H]-ADP, and almost no displacement was observed with UTPγS (Figure 3A) and AA-UTP (data not shown).
Therefore, it was investigated whether UTP analogues could reverse ADP-induced reduction in pVASP-Ser157 (Figure 3B). Indeed, 4γ-UTP but not by UTPγS could reverse ADP-induced reduction in pVASP-Ser157. pVASP-Ser157 in the presence of PGE1-was taken as 1. * p < 0.05 vs. PGE1 alone, § p < 0.05 vs. PGE1 plus ADP.

Antagonism at P2Y12 receptor by UTP thio-analogues was further investigated by directly measuring cAMP production in platelets. Production of cAMP was induced by addition of prostaglandin E1 (PGE1; 1 µM), which was inhibited by the addition of ADP (10 µM). ADP-induced inhibition of cAMP was abrogated by UTP, 25-UTP, and 4S-UTP but not by UTPγS (Figure 3B). Increased levels of cAMP induced phosphorylation of VASP at Ser157 via activation of protein kinase A (PKA) [16] and activation of P2Y12 receptor antagonises the PGE1-mediated VASP phosphorylation. Therefore, it was investigated whether UTP analogues could reverse ADP-induced reduction in

Figure 3. (A) Competitive binding of [3H]-ADP to platelet P2Y12 receptor. Platelets were incubated with 10 nM of [3H]-ADP in the absence (C; control) or presence of saturated concentrations (5x the respective IC50 values) of UTP, 25-UTP, 4S-UTP, UTPγS and binding of [3H]-ADP to P2Y12 receptor was analysed as described in Section 4.4. AR-C66096 (AR-C; 10 µM; a potent P2Y12 receptor antagonist) was used as positive control. The data are means ± S.E.M of three experiments using independent platelet preparations. * p < 0.05 vs. control. n.s.: not significantly different from control; (B) Effect of UTP thio-analogues on cAMP level in PGE1- and ADP-stimulated human platelets. Different preparations of washed platelets were pre-incubated with PGE1 (1 µM) and UTP analogues (UTP 250 µM; 25-UTP 100 µM; 4S-UTP 25 µM; and UTPγS 100 µM) for 10 min as indicated followed by stimulation with ADP (10 µM). * p < 0.05 vs. control, # p < 0.05 vs. PGE1, § p < 0.05 vs. ADP; (C) Effect of 4S-UTP on PGE1-mediated VASP phosphorylation (pVASP-Ser157) in ADP-stimulated human platelets. PRP was pre-incubated with vehicle (resting) or PGE1 (1 µM) followed by treatment with ADP (10 µM) or ADP plus 4S-UTP (25 µM) and pVASP-Ser157 was measured by flow cytometry. pVASP-Ser157 in the presence of PGE1 was taken as 1. * p < 0.05 vs. PGE1 alone, § p < 0.05 vs. PGE1 plus ADP.
PGE₁-mediated VASP phosphorylation. In this context, experiments were performed only with the most active agent, 4S-UTP. Indeed, 4S-UTP could reverse ADP-induced reduction in PGE₁-mediated VASP phosphorylation (Figure 3C).

2.3. Effect of UTP Thio-Analogues on ADP-Induced Platelet Shape Change

Platelet P2Y₁ receptor is responsible for ADP-induced platelet shape change and is partly involved in ADP-induced platelet aggregation. Therefore, we analysed whether UTP thio-analogues also inhibit P2Y₁ receptor activation. ADP-induced platelet shape change was used as a measure of P2Y₁ receptor activation. As shown in Figure 4, ADP (1 µM) induced a significant platelet shape change that was abrogated by the specific P2Y₁ receptor antagonist MRS2500 but not by any of the UTP thio-analogues tested.

![Figure 4](image-url)

**Figure 4.** Effect of UTP thio-analogues on ADP-induced platelet shape change. Quantification of the platelet shape change data from three independent experiments. Platelet were pre-incubated with vehicle or MRS2500 (P2Y₁ receptor antagonist; 1 µM), UTP (250 µM), 2S-UTP (100 µM), 4S-UTP (25 µM), or UTPγS (100 µM) and then treated with ADP (1 µM). *p < 0.05 vs. ADP alone.

3. Discussion

The main and novel finding of the present study is that UTP thio-analogues 2S-UTP and 4S-UTP are potent antagonists of ADP- as well as collagen-induced platelet aggregation. The data from receptor binding and cAMP assays demonstrate that this inhibition is due to antagonism of P2Y₁₂ receptors.

P2Y receptors are a class of purinergic G-protein-coupled receptors activated by naturally occurring extracellular nucleotides. In humans, eight P2Y receptors, namely P2Y₁, P2Y₂, P2Y₄, P2Y₆, and P2Y₁₁–P2Y₁₄, have been identified [17,18]. These P2Y receptors have variable affinity towards different natural nucleotides and nucleosides, e.g., ADP acts as a selective agonist for P2Y₁ and P2Y₁₂ receptors [19], but in contrast, ATP is a potent antagonist for both of these receptors [20,21] and an agonist for P2Y₂ and P2Y₁₁ receptors [22]. Similarly, UTP is a known natural agonist for P2Y₂ and P2Y₄ receptors [13,15], and UDP activates both P2Y₆ and P2Y₁₄ receptors [15,19].

Platelet aggregation is a complex process involving multiple receptors and signalling pathways. Platelet ADP receptors, particularly P2Y₁₂ receptor, play a crucial role in agonist-induced platelet aggregation and thrombus formation [6,23]. Therefore, P2Y₁₂ receptor is of particular interest in the quest to develop novel anti-thrombotic molecules for therapeutic interventions [12].
of platelet P2Y12 receptor by ADP causes a reduction in platelet cAMP content, leading to platelet aggregation [7,21]. The data of the present study demonstrate that UTP derivatives, especially 2’S- and 4’S-UTP, potently antagonise ADP-induced reduction in cAMP and VASP phosphorylation, consistent with an inhibition of the P2Y12 receptor. This was further confirmed by P2Y12 receptor binding analyses. Activation of platelet P2Y1 receptor triggers Ca2+ mobilisation from the platelet dense tubular system and shape change [9,10]. Using platelet shape change as a measure of P2Y1 receptor activation we demonstrate that UTP and all of the analogues tested showed no antagonistic activity at this receptor. Since nucleotides and nucleosides are natural ligands for purinergic receptors, structural modification of these compounds can be exploited to develop novel agonists and antagonists. For example, ADP is a natural ligand for P2Y12 receptor with an EC50 of 1.26 µM, and a thio-methyl (–SCH3) derivative of ADP (2MeSADP) is ~2000-fold more potent than ADP [24]. On the other hand ATP is an endogenous antagonist for P2Y12 receptor, and its thio-derivative is a highly potent P2Y12 receptor antagonist, cangrelor, that has recently been approved for clinical use [22]. UTP is a natural ligand for P2Y2 and P2Y4 receptors [13,15], and different derivatives of UTP have been developed to enhance the stability as well as affinity and specificity of the nucleotide towards these receptors. 2-Thio modification of UTP preserves its potency at the P2Y2 receptor and a further 2′-deoxy-2′-amino modification in the ribose ring further enhances potency and selectivity [17]. Similarly, thiol substitution of oxygen at position 4 resulted in a 4-fold increase in its agonist activity at the P2Y2 receptor [15]. In the present study, UTP was identified as a weak P2Y12 receptor antagonist; however, substitution of oxygen at either position 2 or 4 of the pyrimidine ring results in greatly enhanced antagonist activity at the P2Y12 receptor (with 4-thio substitution being more potent than 2-thio). Addition of a hydrophobic iso-butyl group to the 4-SH group, however, results in reduction in its activity. Addition of a –SH moiety to γ-phosphate of the sugar moiety results in an enzymatically stable compound [25] but this causes a strong reduction in its pEC50 from 8.10 (UTP) to 6.62 (UTPγS) as an agonist at the P2Y2 receptor [15,17]. Accordingly, UTPγS was devoid of antagonist activity at the P2Y12 receptor in the present study. Addition of an alkyl group at position 5 of the pyrimidine base resulted in reduction in its agonist activity at P2Y2 receptor [15]. This phenomenon was also observed in the present study, where addition of an amino-alkyl group at this position resulted in complete loss of its anti-platelet aggregation activity. Thiol modification of positions 2 and 4 of the pyrimidine ring in UTP possibly results in enhanced interaction of the nucleotide with cysteine residues of the P2Y12 receptor, resulting in enhanced antagonist activity. This assumption is based on previous studies demonstrating that active metabolites of both clopidogrel and prasugrel interact with extracellular cysteine residues of the P2Y12 receptor, which appears to be one the mechanisms of their receptor antagonism [26,27]. The present study is carried out on PRP, which although contains the major components of the platelet aggregation system but still lacks the cellular components of the blood. Therefore, IC50 values calculated in the PRP may differ from the whole blood aggregation studies.

4. Materials and Methods

4.1. Materials

PGE1 was from Biomol (Hamburg, Germany), anti-phospho-VASP (Ser157) antibody was from Cell Signaling (Danvers, MA, USA), Adenosine diphosphate (ADP) was from Enzo Life Science (Lörrach, Germany), 2-thiouridine 5′-triphosphate (2S-UTP), 4-thiouridine 5′-triphosphate (4S-UTP), 4-thio-isobutyl uridine 5′-triphosphate (4S-ib-UTP) was from Jena Bioscience (Jena, Germany), Collagen was from Nycomed (Linz, Austria), uridine triphosphate (UTP), UTPγS, and ARL 67156 were from Sigma (Steinheim, Germany). All other chemicals were of the best available quality, usually analytical grade.
4.2. Platelet-Rich Plasma (PRP) and Washed Platelet Preparation

The study conforms to the principles outlined in the “Declaration of Helsinki” (Cardiovascular Research 1997; 35: 2–3) for the use of human material. After approval from the local ethics committee of the University of Giessen (Giessen, Germany), peripheral blood was obtained from healthy human (male and female) volunteers (20–45 years old) who had not taken any drugs for at least 14 days. Blood samples were drawn into tubes containing trisodium citrate (Sarstedt, Germany). Whole blood was centrifuged at 110 × g for 20 min at room temperature (RT) to obtain PRP. The platelet content was measured using an automatic haematology analyser Sysmex KX-21 (Sysmex, Germany). Platelet-poor plasma (PPP) was obtained by centrifugation of PRP at 14,000 × g for 3 min. The platelet count in PRP was adjusted to (250–280) × 10^6/mL by diluting native PRP with the same donor’s PPP.

In order to obtain washed platelets, the PRP was centrifuged at 600 × g for 20 min at RT. The platelet pellet was re-suspended in Tyrode’s buffer (pH 7.2) containing PGI2 (0.5 µM) and albumin (0.1%) and the suspension was re-centrifuged at 600 × g for 10 min. Finally, the washed platelets were re-suspended in Tyrode’s buffer (pH 7.2) at the concentration of 3 × 10^8/mL. The suspended platelets showed a characteristic shimmering effect.

4.3. Platelet Aggregation and Shape Change

Platelet aggregation was measured with two-channel Chrono-Log aggregometer (Chrono-Log Corporation, Havertown, PA, USA) at 37 °C using stirred (1000 rpm) PRP. Various concentrations of agonists and/or antagonists as indicated in the figures or legends were added in a total volume of 50 µL NaCl (0.9%) solution for a final volume 500 µL. The relative platelet aggregation response to ADP was determined by comparison of light transmission through PPP (500 µL) and is expressed as a percentage response. UTP and its analogues were added 1 min prior to ADP addition.

The platelet shape change was measured using the offset mode of the Aggro/Link computer interface. The reference cuvette of the aggregometer contained a platelet suspension equivalent to 50% of the test samples (to amplify the signal). Abciximab (2 µg/mL) was added to all the samples to prevent platelet aggregation and obtain a stable shape change. The platelet shape change was monitored for 6–10 min after the addition of the agents.

4.4. [3H]-ADP P2Y12 Receptor Binding Assay

Binding of UTP analogues to P2Y12 receptor was determined by displacement of the binding of [3H]-ADP (PerkinElmer, Rodgau, Germany) to platelet P2Y12 receptor according to the protocol described by Savi et al. (2004) [28] using washed human platelets. Experiments were carried out in triplicate in a total volume of 100 µL Tyrode’s buffer (pH 7.2) containing 0.5 × 10^6 platelets/µL and 10 nM [3H]-ADP at RT. The binding assays were performed in the presence of a P2Y1 receptor antagonist MRS2500 (10 µM). Non-specific binding was defined as the binding of [3H]-ADP measured in the presence of a saturating concentration of nonradioactive ADP (1 mM). For competitive binding of UTP analogues to P2Y12 receptor, the analogues were added at concentrations 5-fold higher than their respective IC50 values to obtain complete receptor saturation. The [3H]-ADP radioactivity was measured using an LS6500 (Beckman Coulter, Krefeld, Germany) automatic liquid scintillation counter.

4.5. Platelet cAMP Assay

cAMP levels were measured in washed platelets (100 µL) using a chemiluminescence-based HitHunter cAMP kit (DiscoveRx, Birmingham, UK) according to the manufacturer’s protocol. Briefly, washed platelets (100 µL) were incubated with prostaglandin E1 (PGE1, 1 µM) for 10 min in a 96-well plate. Subsequently, the platelets were stimulated with ADP (10 µM) in the absence or presence of UTP and its thio-analogues for 5 min. The reaction was stopped by adding stop buffer to the platelets and following the protocol outlined by the manufacturer. The cAMP content of each well was determined.
using an “Infinite® 200” multi-plate reader (Tecan, Männedorf, Switzerland). Levels of cAMP were normalized to the platelet count used for the measurements.

4.6. Platelet VASP Phosphorylation

The platelet intracellular VASP-phosphorylation was determined by flow cytometry using anti-phospho-VASP (Ser157) antibody. PRP was incubated with vehicle or PGE_1 (1 µM) for 10 min followed by the addition of ADP (10 µM) or 4S-UTP (25 µM) plus ADP and incubated for additional 5 min. Thereafter the platelets were fixed in 3% para formaldehyde for 10 min at room temperature (RT) and permeabilised with 0.2% triton X-100 for 10 min at RT and incubated with anti-phospho VASP antibody. After 30 min of incubation, platelets were washed in PBS and incubated with secondary FITC conjugated polyclonal anti-rabbit IgG antibody (DL488; Thermo fisher scientific, Dreieich, Germany) and analysed by FACS (Becton Dickinson, Heidelberg, Germany).

4.7. Data Presentation and Statistics

Each experiment was repeated at least 3 times. The data are presented as representative aggregometer tracings from a typical experiment. Dose–response curves were generated using Graphpad Prism software (Graphpad Software Inc., San Diego, CA, USA) from means ± S.E.M of transformed (i.e., percentage control) data pooled from 3 different experiments. The IC_{50} value for each agent was determined from 3 different concentrations of the agent using the Schild analysis function of Graphpad Prism.

5. Conclusions

UTP and its thio-derivatives are known to be potent agonists for P2Y_2 and P2Y_4 receptors [13,15]. Here, we describe for the first time that the thio-derivatives of UTP act as potent antagonists for platelet P2Y_{12} receptor. Addition of a thiol group at position 4 of the uracil moiety results in a highly potent P2Y_{12} receptor antagonist. Future studies exploiting modifications of this uracil position may provide even more potent reversible acting P2Y_{12} receptor antagonists that may be employed clinically to reduce thrombotic events.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

| Abbreviation | Description                      |
|--------------|----------------------------------|
| ATP          | Adenosine 5'-triphosphate        |
| ADP          | Adenosine 5'-diphosphate         |
| UTP          | Uridine 5'-triphosphate          |
| 2S-UTP       | 2-Thiouridine 5'-triphosphate    |
| 4S-UTP       | 4-Thiouridine 5'-triphosphate    |
| 4S-ib-UTP    | 4-Thio-isobutyl uridine 5'-triphosphate |
| AA-UTP       | 5-Amino allyl uridine 5'-triphosphate |
Appendix A. Chemical Compounds Studied in This Article

ADP (PubChem CID: 6022); UTP (PubChem CID: 6133); 2S-UTP (PubChem CID: 1017453); 4S-UTP (PubChem CID: 3033941); 4S-ib-UTP (PubChem CID: Not available); AA-UTP (PubChem CID: 16218928); and UTPγS (PubChem CID: 5311494).

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