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Article

Uridine Triphosphate Thio-Analogues Inhibit Platelet P2Y12 Receptors and Aggregation

Condensed title: UTP thio-analogues and platelet aggregation,

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Abstract: Platelet P2Y12 is an important 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 P2Y12 but not P2Y1 receptors and antagonises 10 µM ADP-induced platelet aggregation in a concentration-dependent manner with an IC_{50} value of ~250 µM. An 8-fold higher platelet inhibitory activity was observed with a 2-thio analogue of UTP (2S-UTP), with an IC_{50} of 30 µM. The 4-thio analogue (4S-UTP) with an IC_{50} of 7.5 µM was 33-fold more effective. A 3-fold decrease in inhibitory activity, however, was observed by introducing an isobutyl group at the 4S- position. A complete loss of inhibition was observed with thio-modification of the γ phosphate of the sugar moiety, which yields an enzymatically stable analogue. The interaction of UTP analogues with P2Y12 receptors was verified by P2Y12 receptor binding and cAMP assays. These novel data demonstrate for the first time that 2- and 4-thio analogues of UTP are potent P2Y12 receptor antagonists that may be useful for therapeutic intervention.

Keywords: 2S-UTP; 4S-UTP; P2Y12 receptors; 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 P2Y1 and P2Y12 that are activated by adenosine diphosphate (ADP) and play a central role in platelet activation and aggregation [5-8]. Both P2Y1 and P2Y12 receptors are G-protein-coupled receptors that are coupled to G_q and G_i, respectively [7]. P2Y1 receptor activation triggers Ca^{2+} mobilisation from the platelet dense tubular system and shape

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change [9;10], whereas activation of $P2Y_{12}$ receptors causes an inhibition of adenylyl cyclase-dependent cyclic AMP (cAMP) production and platelet aggregation [7;11]. Because of its central role in thrombus formation and stabilisation, the $P2Y_{12}$ receptor is a well-established target for anti-thrombotic drug development [12]. Presently, only a few $P2Y_{12}$ 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 $P2Y_{12}$ receptor antagonists. Currently used $P2Y_{12}$ 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 $P2Y_{12}$ receptors and thus inhibit their activation. Uridine triphosphate (UTP) [13;14] and its thio-derivatives [15] act as natural and synthetic ligands for purinergic $P2Y_2$ and $P2Y_4$ receptors, respectively. The present study analysed the anti-platelet aggregation activity of UTP and a number of different thio-analogues, including UTPγS, aminoallyl UTP (AA-UTP), 2-thio UTP (2S-UTP), 4-thio UTP (4S-UTP), and 4-thio-isobutyl UTP (4S-*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 UTP thio-analogues antagonise ADP-induced platelet aggregation with various potencies

Fig. 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 µM caused 75-90% platelet aggregation as measured by change in the turbidity of PRP (Fig. 2). UTP antagonised ADP-induced platelet aggregation weakly with an IC_{50} value of ~250 µM (Table 1). The 2-thio derivative of UTP (2S-UTP; compound 2) displayed a stronger inhibition of platelet aggregation (Fig. 2A) with an IC_{50} of ~30 µM (Table 1). There was a 33-fold increase (vs. UTP) in platelet inhibition by thio (-S-) modification of -O- at position 4 of the pyrimidine ring. This compound (4S-UTP) was able to inhibit ADP-induced platelet aggregation completely at 15 µM (Fig. 2B) with an IC_{50} of ~7.5 µM. Addition of an isobutyl group at the 4S position to yield 4S-*ib*-UTP, however, resulted in a 3-fold decrease in inhibitory activity (Fig. 2C). The non-hydrolysable stable analogue of UTP, UTPγS, showed very little activity against ADP-induced platelet aggregation (Fig. 2D). Likewise, the aminoallyl derivative of UTP (AA-UTP) was virtually devoid of any anti-platelet activity. The IC_{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 $P2Y_{12}$ receptors which are 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,

4S-UTP. Indeed 4S-UTP was able to strongly antagonise the collagen-induce platelet aggregation (Fig. 2E).

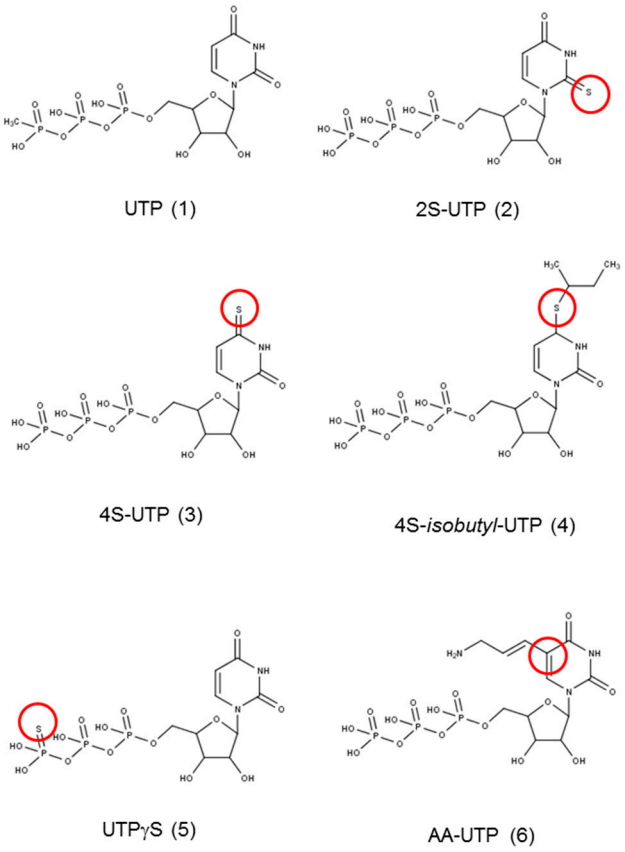

Figure 1. Structure of the compounds used in the study. The marked site shows the modification of the parent compound UTP.

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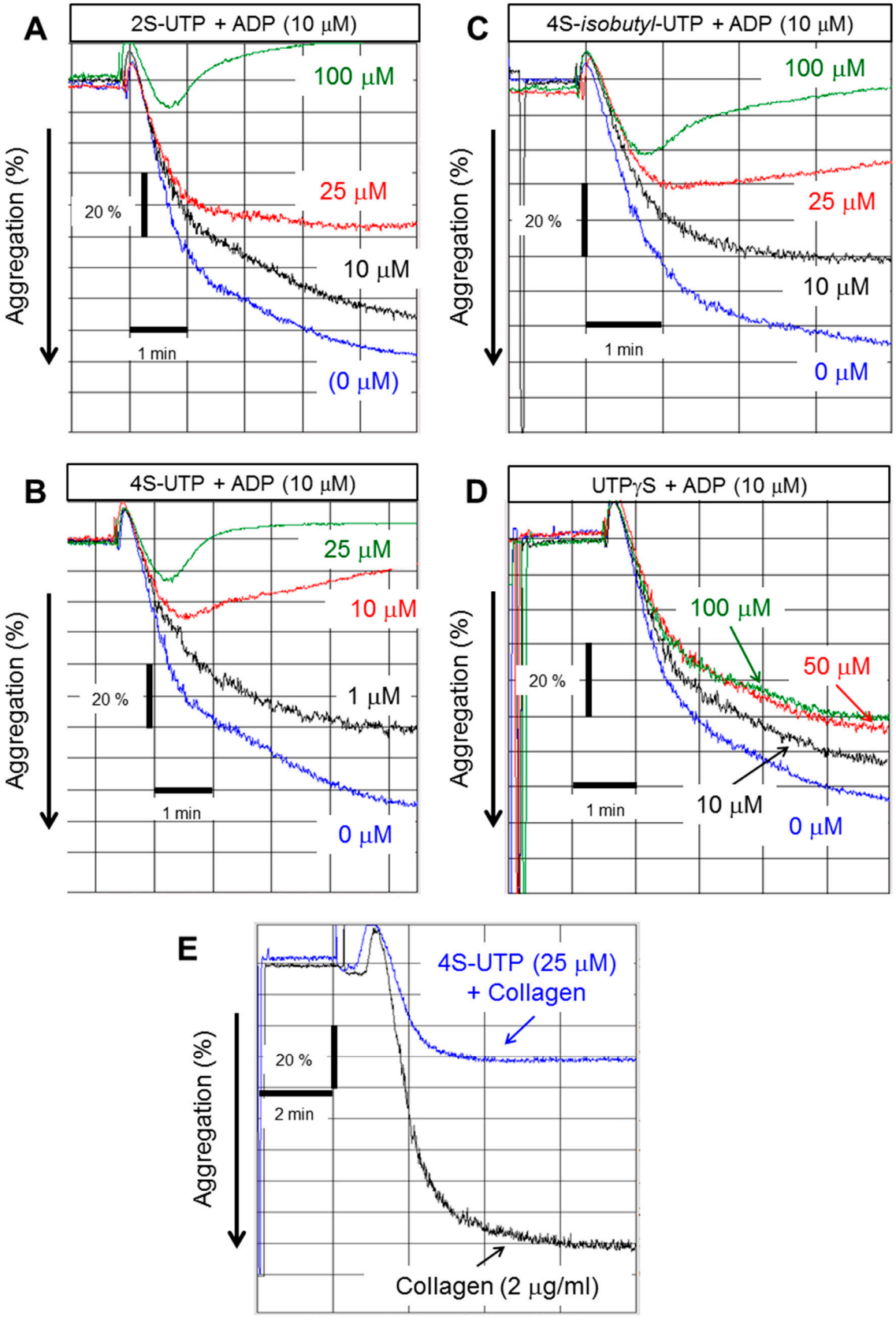

Figure 2. UTP thio-analogues antagonise ADP-induced platelet aggregation. Representative tracing of platelet aggregation induced by ADP (10 µM) in the absence or presence of 2S-UTP **(A),** 4S-UTP **(B),** 4S-isobutyl UTP **(C),** and UTPγS **(D).** Representative tracings from 4 experiments using

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).

Table 1:

Compound No.	Ligand	IC_{50} (μM)
1	UTP	250
2	2S-UTP	30
3	4S-UTP	7.5
4	4S-*ib*-UTP	23
5	UTPγS	>1000
6	AA-UTP	>1000

2.2 Binding of UTP thio-analogues to $P2Y_{12}$ receptors, cAMP production, and vasodilator-stimulated phosphoprotein (VASP) phosphorylation

ADP-induced platelet aggregation is mainly dependent on activation of platelet $P2Y_{12}$ receptors. In order to confirm that UTP thio-analogue-mediated inhibition of ADP-induced platelet aggregation is due to antagonism at $P2Y_{12}$ receptors, radioligand-displacement assays were performed using [^{3}H]-ADP as the $P2Y_{12}$ receptor agonist in the presence of $P2Y_1$ antagonist (MRS2500 10 μM). As shown in Fig. 3A, saturating concentrations of UTP thio-analogues competitively antagonised the binding of [^{3}H]-ADP to $P2Y_{12}$ receptors. 4S-UTP showed the strongest displacement of [^{3}H]-ADP, and almost no displacement was observed with UTPγS (Fig. 3A) and AA-UTP (data not shown).

Antagonism at $P2Y_{12}$ receptors by UTP thio-analogues was further investigated by directly measuring cAMP production in platelets. Production of cAMP was induced by addition of PGE_1 (1 μM), which was inhibited by the addition of ADP (10 μM). ADP-induced inhibition of cAMP was abrogated by UTP, 2S-UTP, and 4S-UTP but not by UTPγS (Fig. 3B). Increased levels of cAMP induce phosphorylation of VASP at Ser157 via activation of PKA [16] and activation of $P2Y_{12}$ receptors antagonises the PGE_1-mediated VASP phosphorylation. Therefore, it was investigated whether UTP analogues could reverse ADP-induced reduction in PGE_1-mediated VASP phosphorylation. In this context experiments were performed only with the most active agent, 4S-UTP. Indeed 4S-UTP was able to reverse ADP-induced reduction in PGE_1-mediated VASP phosphorylation (Fig. 3C).

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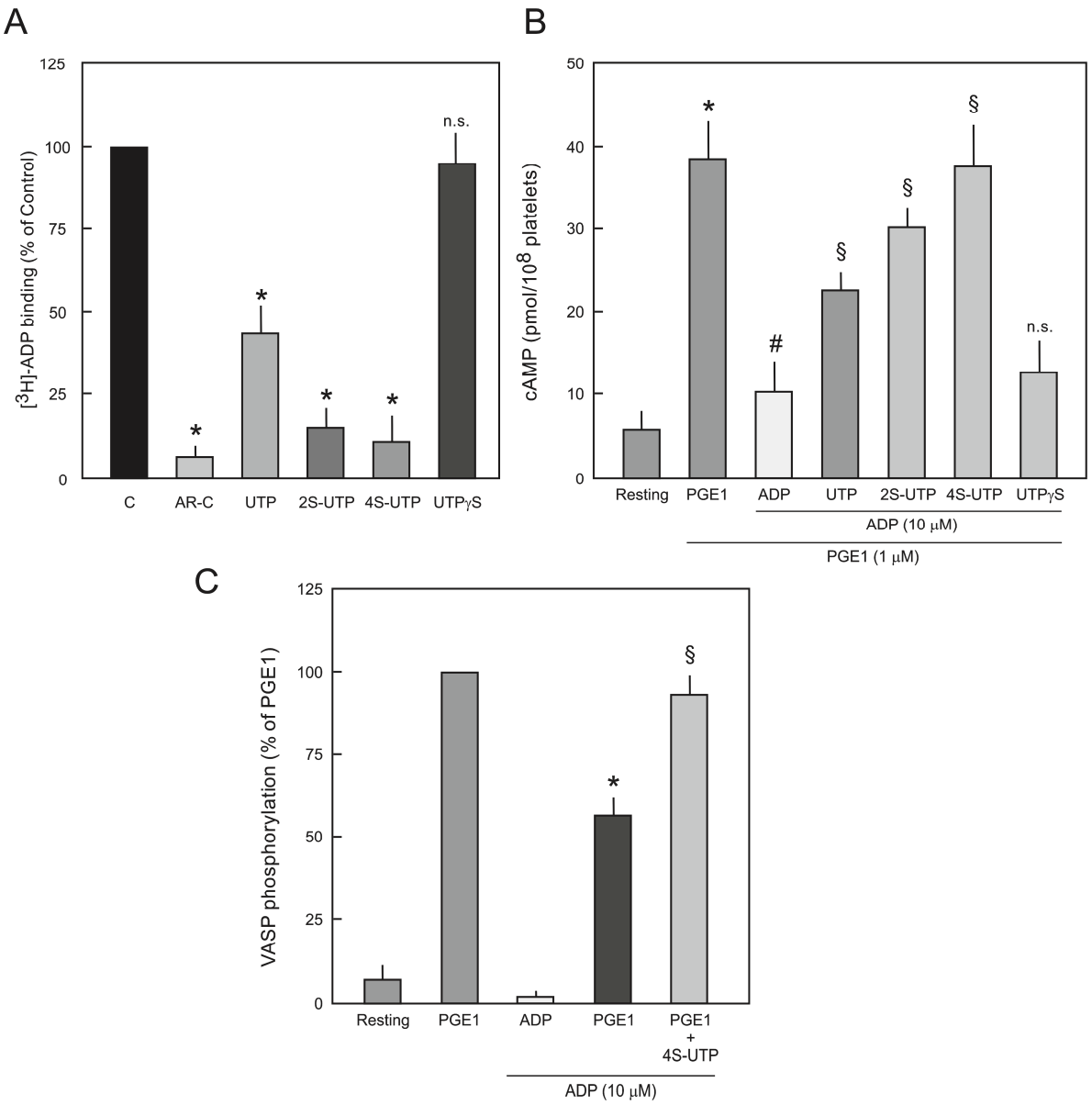

Figure 3. (A) Competitive binding of [³H]-ADP to platelet P2Y₁₂ receptors. Platelets were incubated with 10 nM of [³H]-ADP in the absence (C; control) or presence of saturated concentrations (5x the respective IC_{50} values) of UTP, 2S-UTP, 4S-UTP, UTPγS and binding of [³H]-ADP to P2Y₁₂ receptors was analysed as described in Materials and Methods. AR-C66096 (10 μM; a potent P2Y₁₂ receptor antagonist) was used as positive control. The data ± S.E.M of 3 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 PGE_1 (1 μM) and UTP analogues (UTP 250 μM; 2S-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. PGE_1, $^{\S}p < 0.05$ vs. ADP. **(C) Effect of 4S-UTP on PGE₁-mediated VASP phosphorylation in ADP-stimulated human platelets.** PRP was pre-incubated with vehicle (resting) or PGE_1 (1 μM) followed by treatment with APD (10 μM) or ADP plus 4S-UTP (25 μM) and VASP phosphorylation was measured by flow cytometry. VASP

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phosphorylation in the presence of PGE_1-was taken as 100%. $^{*}p < 0.05$ vs. PGE_1 alone, $^{§}p < 0.05$ vs. PGE_1 plus ADP

2.3. Effect of UTP thio-analogues on ADP-induced platelet shape change

Platelet $P2Y_1$ receptors are responsible for ADP-induced platelet shape change and are partly involved in ADP-induced platelet aggregation. Therefore, we analysed whether UTP thio-analogues also inhibit $P2Y_1$ receptor activation. ADP-induced platelet shape change was used as a measure of $P2Y_1$ receptor activation. As shown in Fig. 4, ADP (1 µM) induced a significant platelet shape change that was abrogated by the specific $P2Y_1$ receptor antagonist MRS2500 but not by any of the UTP thio-analogues tested.

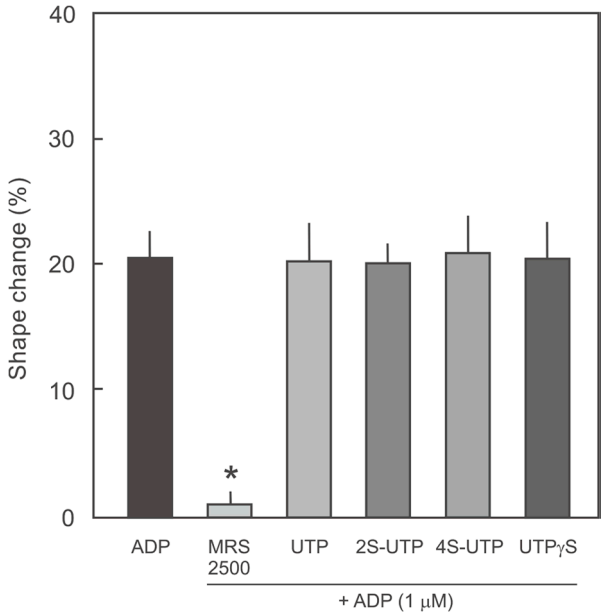

Figure 4. Effect of UTP thio-analogues on ADP-induced platelet shape change. Quantification of the platelet shape change data from 3 independent experiments. Platelet were pre-incubated with vehicle or MRS2500 ($P2Y_1$ receptor antagonist; 1 µM), UTP (250 µM), 2S-UTP (100 µM), 4S-UTP (25 µM), or UTPγS (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 $P2Y_{12}$ receptors.

P2Y receptors are a class of purinergic G-protein-coupled receptors activated by naturally occurring extracellular nucleotides. In humans, eight P2Y receptors, namely $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$-$P2Y_{14}$, 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 $P2Y_1$ and $P2Y_{12}$

receptors [19], but in contrast, ATP is a potent antagonist for both of these receptors [20;21] and an agonist for $P2Y_2$ and $P2Y_{11}$ receptors [22]. Similarly, UTP is a known natural agonist for $P2Y_2$ and $P2Y_4$ receptors [15;23], and UDP activates both $P2Y_6$ and $P2Y_{14}$ receptors [15;19].

Platelet aggregation is a complex process involving multiple receptors and signalling pathways. Platelet ADP receptors, particularly $P2Y_{12}$ receptors, play a crucial role in agonist-induced platelet aggregation and thrombus formation [6;24]. Therefore, $P2Y_{12}$ receptors are of particular interest in the quest to develop novel anti-thrombotic molecules for therapeutic interventions [12]. Activation of platelet $P2Y_{12}$ 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 2S- and 4S-UTP, potently antagonise ADP-induced reduction in cAMP and VASP phosphorylation, consistent with an inhibition of the $P2Y_{12}$ receptor. This was further confirmed by $P2Y_{12}$ receptor binding analyses. Activation of platelet $P2Y_1$ receptor triggers Ca^{2+} mobilisation from the platelet dense tubular system and shape change [9;10]. Using platelet shape change as a measure of $P2Y_1$ 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 $P2Y_{12}$ receptors with an EC_{50} of 1.26 μM, and a thio-methyl ($-SCH_3$) derivative of ADP (2MeSADP) is ~2000-fold more potent than ADP [25]. On the other hand ATP is an endogenous antagonist for $P2Y_{12}$ receptors, and its thio-derivative is a highly potent $P2Y_{12}$ receptor antagonist, cangrelor, that has recently been approved for clinical use [22]. UTP is a natural ligand for $P2Y_2$ and $P2Y_4$ receptors [15;23], 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 $P2Y_2$ 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 $P2Y_2$ receptor [15]. In the present study, UTP was identified as a weak $P2Y_{12}$ receptor antagonist; however, substitution of oxygen at either position 2 or 4 of the pyrimidine ring results in greatly enhanced antagonist activity at the $P2Y_{12}$ 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 [26] but this causes a strong reduction in its pEC_{50} from 8.10 (UTP) to 6.62 (UTPγS) as an agonist at the $P2Y_2$ receptor [15;17]. Accordingly, UTPγS was devoid of antagonist activity at the $P2Y_{12}$ 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 $P2Y_2$ 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 $P2Y_{12}$ 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 $P2Y_{12}$ receptor, which appears to be one the mechanisms of their receptor antagonism [27;28]. 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 IC_{50} values calculated in the PRP may differ from the whole blood aggregation studies.

4. Materials and Methods

4.1. Materials:

PGE_1 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, 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 x *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 x *g* for 3 min. The platelet count in PRP was adjusted to 250-280 x 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 x g for 20 min at RT. The platelet pellet was re-suspended in Tyrode's buffer (pH 7.2) containing PGI_2 (0.5 μM) and albumin (0.1 %) and the suspension was re-centrifuged at 600 x *g* for 10 min. Finally, the washed platelets

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were re-suspended in Tyrode's buffer (pH 7.2) at the concentration of 3 x 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, 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. [^{3}H]-ADP $P2Y_{12}$ receptor binding assay:

Binding of UTP analogues to $P2Y_{12}$ receptors was determined by displacement of the binding of [^{3}H]-ADP (PerkinElmer) to platelet $P2Y_{12}$ receptors according to the protocol described by Savi et al. (2004) 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 x 10^6 platelets/µl and 10 nM [^{3}H]-ADP at RT. The binding assays were performed in the presence of a $P2Y_1$ receptor antagonist MRS2500 (10 µM). Non-specific binding was defined as the binding of [^{3}H]-ADP measured in the presence of a saturating concentration of nonradioactive ADP (1 mM). For competitive binding of UTP analogues to $P2Y_{12}$ receptors, the analogues were added at concentrations 5-fold higher than their respective IC_{50} values to obtain complete receptor saturation. The [^{3}H]-ADP radioactivity was measured using an LS6500 (Beckman Coulter) 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 E_1 (PGE_1, 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

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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 minutes 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 minutes of incubation, platelets were washed in PBS and incubated with secondary FITC conjugated polyclonal anti-rabbit IgG antibody (DL488; Thermo fisher scientific, 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. Perspectives/Conclusion:

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 $P2Y_{12}$ receptors. 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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Author contribution: D. Gündüz conceived and designed the experiments and critically reviewed the manuscript. C. Tanislav, D. Sedding, M. Parahuleva, C. Troidl, S. Santoso, and C. Hamm contributed reagents,

analysed data, and critically reviewed the manuscript. M. Aslam conceived, designed, and performed the experiments, analysed data, and wrote the manuscript.

Conflict of interest: None

Abbreviations:

ATP:	adenosine 5′-triphosphate
ADP:	adenosine 5′-diphosphate
UTP:	uridine 5′-triphosphate
2S-UTP:	2-thio uridine 5′-triphosphate
4S-UTP:	4-thio uridine 5′-triphosphate
4S-*ib*-UTP:	4-thio isobutyl uridine 5′-triphosphate
AA-UTP:	5-amino allyl uridine 5′-triphosphate

Chemical compounds studied in this article

ADP (PubChem CID: 6022); UTP (PubChem CID: 6133); 2S-UTP (PubChem CID: 10174453); 4S-UTP (PubChem CID: 3033941); 4S-*i-butyl*-UTP (PubChem CID: Not available); AA-UTP (PubChem CID: 16218928); and UTPγS (PubChem CID: 5311494)

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