 doi:10.20944/preprints201611.0048.v1

Article

Uridine Triphosphate Thio-analogues Inhibit Platelet $P2Y_{12}$ Receptors and Aggregation

Condensed title: UTP Thio-analogues and Platelet Aggregation

Dursun Gündüz [1], Christian Tanislav [2], Daniel Sedding [3], Mariana Parahuleva [4], Sentot Santoso [5], Christian Troidl [1], Christian Hamm [1], Muhammad Aslam [1,*]

[1] Department of Cardiology/Angiology, University Hospital Giessen, 35392 Giessen, Germany
[2] Department of Neurology, University Hospital Giessen, 35392 Giessen, Germany
[3] Department of Cardiology/Angiology, Hannover Medical School, 30625 Hannover, Germany
[4] Department of Cardiology/Angiology, University Hospital, Marburg, 35392 Giessen, Germany
[5] Institute for Clinical Immunology and Transfusion Medicine, Justus Liebig University, 35385 Giessen, Germany
* Correspondence: muhammad.aslam@med.uni-giessen.de; Ph.:+49 641 99 42248 Fax.:+49 641 99 42259

Abstract: Platelet $P2Y_{12}$ is an important ADP receptor that is involved in agonists-induced platelet aggregation and is an important target for the development of anti-platelet aggregation drugs. Here the effects of thio-analogues of uridine triphosphate (UTP) on ADP-induced platelet aggregation are characterised. Using human platelet rich plasma we demonstrate that UTP inhibits $P2Y_{12}$ but not $P2Y_1$ receptors and antagonises ADP-induced platelet aggregation in a conc.-dependent manner with an IC_{50} value of ~250 µM against ADP (10 µM). An 8-fold increase in the platelet inhibitory activity was observed with 2-thio analogue of UTP (2S-UTP) with an IC_{50} value of 30 µM. A 33-fold increase in anti-platelet aggregation activity was observed with 4-thio analogue (4S-UTP) with an IC_{50} value of 7.5 µM. However, a 3-fold decrease in activity was observed by introducing an isobutyl group at the 4S- position. A complete loss in anti-platelet aggregation activity was observed with thio-modification of gamma phosphate of the sugar moiety which yields an enzymatically stable analogue. The interaction of UTP analogues with $P2Y_{12}$ receptors was further verified by $P2Y_{12}$ receptor binding assay and cAMP assay. The novel data demonstrate for the first time that 2- and 4-thio analogues of UTP are potent $P2Y_{12}$ receptor antagonists that can be useful candidates for therapeutic intervention.

Keywords: 2S-UTP; 4S-UTP; $P2Y_{12}$ receptors; ADP; platelet aggregation

Abbreviations:

ATP:	adenosine 5′ triphosphate
ADP:	adenosine 5′ diphosphate
UTP:	uridine 5′ triphosphate

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 November 2016 doi:10.20944/preprints201611.0048.v1

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)

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 anomalies such as myocardial infarction [1;2] and thrombosis [3;4]. Platelets express nucleotide receptors $P2Y_1$ and $P2Y_{12}$ activated by adenosine diphosphate (ADP) and play a central role in platelet activation and aggregation [5-8]. Both $P2Y_1$ and $P2Y_{12}$ receptors are G-protein coupled receptors, and are coupled to G_q and G_i, respectively [7]. $P2Y_1$ receptor activation triggers Ca^{2+} mobilisation from the platelet dense tubular system and shape change [9;10], while activation of $P2Y_{12}$ receptors causes an inhibition of the adenylyl cyclase-dependent 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. Presently, only few $P2Y_{12}$ receptor antagonists (both reversible and irreversible) are available for clinical interventions and because of their pharmacokinetic profile have limitations in clinical practice. Therefore there is a need for the development of novel $P2Y_{12}$ receptor antagonists. Currently used $P2Y_{12}$ receptor antagonists are either pro-drugs requiring to be converted to active metabolites by liver metabolic machinery such as clopidogril or are different nucleotide derivatives such as ticagrelor reversibly binding to $P2Y_{12}$ receptors and thus inhibit their activation. Uridine triphosphate (UTP) [12;13] and its thio-derivatives [14] 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 its different thio-analogues which include UTP, 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 variable 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 turbidity of platelet rich plasma (PRP) (Fig. 2). UTP antagonised ADP-

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 November 2016 doi:10.20944/preprints201611.0048.v1

induced platelet aggregation weakly with an IC_{50} value of ~250 µM (Table 1). The 2-thio derivative of UTP (2S-UTP; compound 2) depicted a strong anti-platelet aggregation activity (Fig. 2A) with an IC_{50} value of ~30 µM (Table 1). There was a 33-fold increase (from UTP) in anti-platelet aggregation activity by thio (-S-) modification of -O- at position 4 of the pyrimidine ring (4S-UTP). This compound (4S-UTP) was able to inhibit the ADP-induced platelet aggregation completely at 15 µM concentration (Fig. 2B) with an IC_{50} value of ~7.5 µM. Surprisingly, addition of an isobutyl group at the 4S (4S-*ib*-UTP) resulted in 3-fold loss in 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.

2.2 Binding of UTP thio-analogues to $P2Y_{12}$ receptors and cAMP production

ADP-induced platelet aggregation is mainly dependent on activation of platelet $P2Y_{12}$ receptors. In order to confirm that UTP thio-analogues-mediated inhibition of ADP-induced platelet aggregation is due to antagonism at $P2Y_{12}$ receptors, ligand-displacement assay was performed using [^{3}H]-ADP as $P2Y_{12}$ receptor agonist. As shown in Fig. 3A, saturating concentrations of UTP thio-analogues competitively antagonised the binding of [^{3}H]-ADP to $P2Y_{12}$ receptors in the presence of $P2Y_1$ antagonist (MRS2500 10 µM). The 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).

Inhibition of $P2Y_{12}$ 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 could be 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).

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, it was 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 which was abrogated by a specific $P2Y_1$ receptor antagonist MRS2500 but not by any of the UTP thio-analogues tested.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 November 2016 doi:10.20944/preprints201611.0048.v1

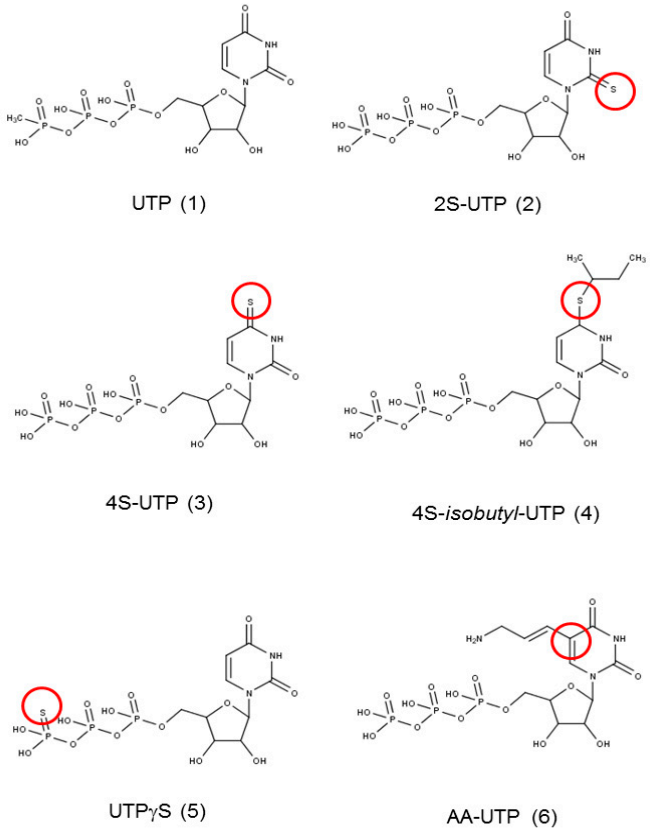

Figure 1. Structure of the compounds used in the study. Marked site is the modification at the parent compound UTP.

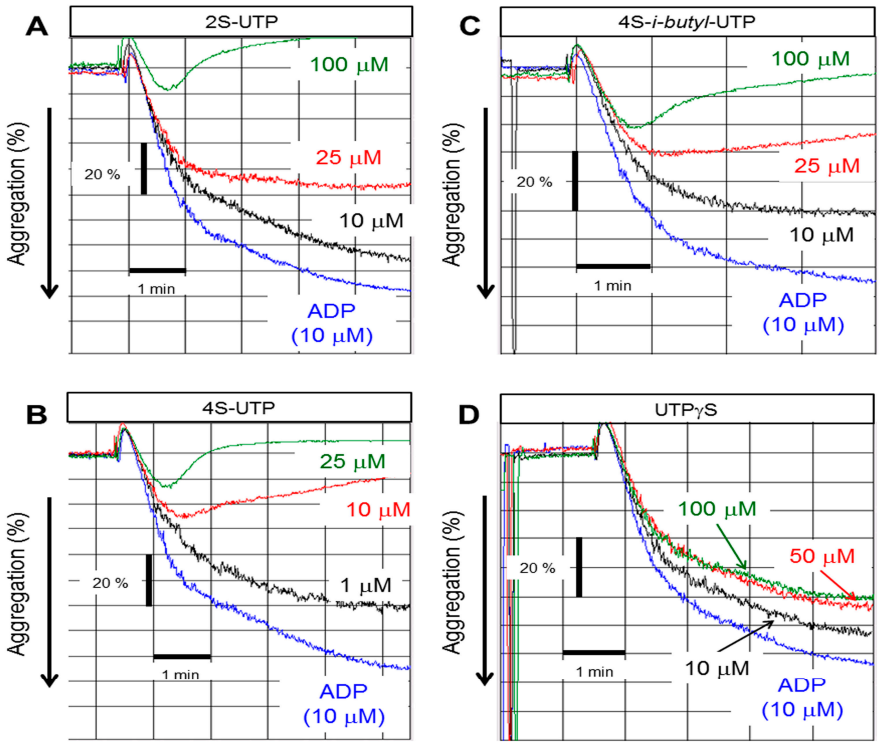

Figure 2. UTP thio-analogues antagonise ADP-induced platelet aggregation. Representative tracing of platelets aggregation induced by ADP (10 μM) in the absence or presence of 2S-UTP **(A),** 4S-UTP **(B),** 4S-isobutyl UTP **(C),** and UTPγS **(D).** Representative tracings from 4 experiments of independent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

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 November 2016 doi:10.20944/preprints201611.0048.v1

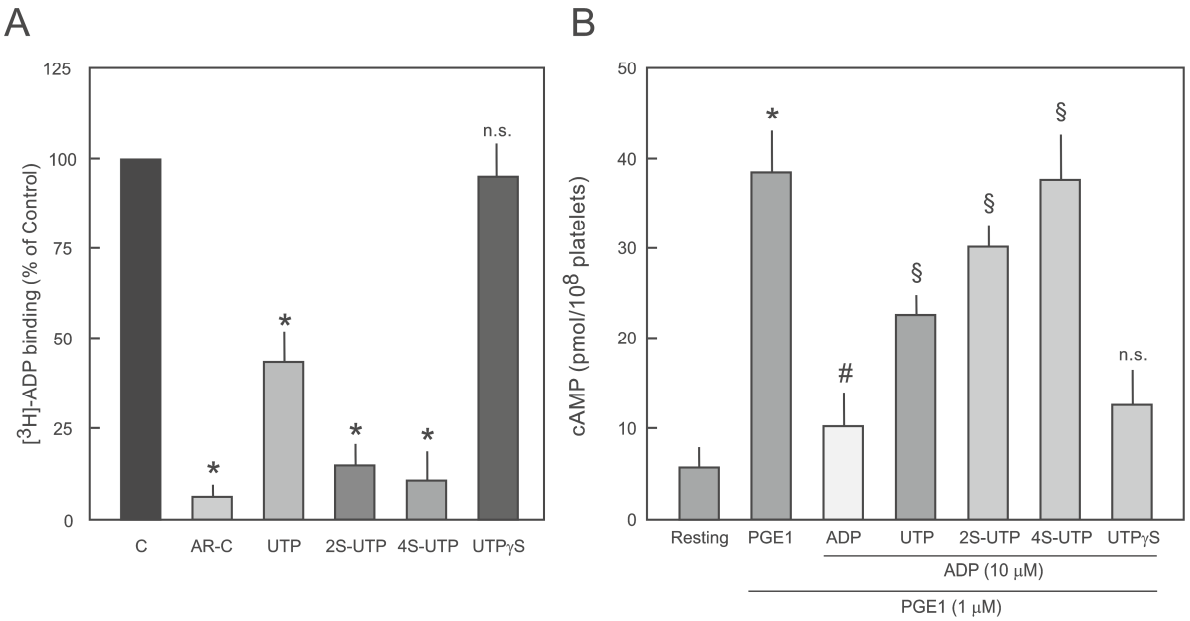

Figure 3. (A) Competitive binding of [^{3}H]-ADP to platelet P2Y$_{12}$ receptors. Platelets were incubated with 10 nM of [^{3}H]-ADP in the absence (C; control) or presence of UTP, 2S-UTP, 4S-UTP, UTPγS and binding of [^{3}H]-ADP to P2Y$_{12}$ receptors was analysed as described in methods. The data ± S.E.M of 3 experiments of 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 PGE$_1$- and ADP-stimulated human platelets.** The platelets from different group pre-incubated with PGE$_1$ (10 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.

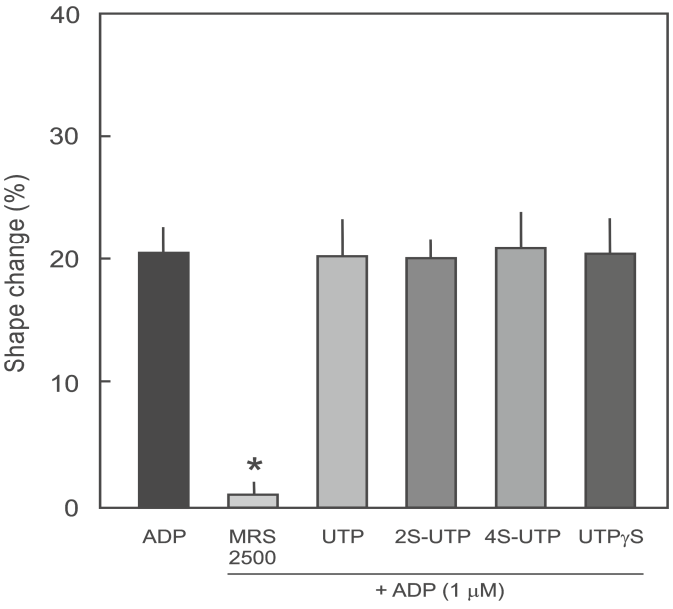

Figure 4. Effect of UTP thio-analogues on ADP-induced platelet shape change. Quantification of the platelet shape change data from 3 independent experiments. Platelet were pre-incubated with vehicle or MRS2500 (P2Y$_1$ receptor antagonist; 1 μM), UTP (250 μM), 2S-UTP (100 μM), 4S-UTP (25 μM), or UTPγS (100 μM) and then treated with ADP (1 μM). $^*p < 0.05$ vs. ADP alone.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 November 2016 doi:10.20944/preprints201611.0048.v1

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	AA-UTP	>1000
6	UTPγS	>1000

3. Discussion

The main and novel finding of present study is that UTP thio-analogues, 2S-UTP and 4S-UTP are potent antagonists of platelet ADP-induced 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 (GPCRs) activated by naturally occurring extracellular nucleotides. In humans, eight P2Y receptors namely $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$-$P2Y_{14}$ have been identified [15]. These P2Y receptors have variable affinity towards different natural nucleotides e.g. ADP acts as a selective agonist for $P2Y_1$ and $P2Y_{12}$ receptors [16], but in contrast, ATP is a potent antagonist for both of these receptors [17;18] and agonist for $P2Y_2$ and $P2Y_{11}$ receptors [19]. Similarly, UTP is a known natural agonist for $P2Y_2$ and $P2Y_4$ receptors [14;20] and UDP activates both $P2Y_6$ and $P2Y_{14}$ receptors [14;16].

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;21]. Therefore, $P2Y_{12}$ receptors are of particular interest to develop novel anti-thrombotic molecules for therapeutic interventions. Activation of platelet $P2Y_{12}$ receptor by ADP causes a reduction in cAMP content leading to platelet aggregation [7;18]. The data of the present study demonstrate that UTP itself weakly, but its 2S- and 4S-derivatives potently antagonised ADP-induced reduction in cAMP suggesting an inhibition of $P2Y_{12}$ 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 are natural ligands for purinergic receptors structural modification of these nucleotides is exploited to develop novel agonists and antagonists for the receptors, e.g. ADP is a natural ligand for $P2Y_{12}$ receptors with EC_{50} of 1.26 μM and a thio-methyl (-SCH_3) derivative of ADP (2MeSADP) is ~2000-fold more potent than ADP [22]. On the other hand ATP is an endogenous antagonist for $P2Y_{12}$ receptors and its structural modification resulted in the development of a highly potent $P2Y_{12}$ receptor antagonist, cangrelor (a thio-derivative), and has recently been

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 November 2016 doi:10.20944/preprints201611.0048.v1

approved for clinical use [19]. UTP is a natural ligand for $P2Y_2$ and $P2Y_4$ receptors [14;20], 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 $P2Y_2$ receptor and a further 2′-deoxy-2′-amino modification in the ribose ring of 2-thio UTP enhances its potency and selectivity to towards $P2Y_2$ receptor [15]. Similarly, substitution of oxygen at 4-position by thiol group resulted in a 4-fold increase in its agonist activity at $P2Y_2$ receptor [14]. In the present study, UTP is identified as a weak antagonist at $P2Y_{12}$ receptor, however, substitution of oxygen either at position 2 or 4 of the pyrimidine ring results in greatly enhanced antagonist activities at $P2Y_{12}$ receptor (4-thio more potent than 2-thio). However, addition of a hydrophobic iso-butyl group to the –SH at 4-position results in reduction in its activity. Addition of a –SH moiety to γ-phosphate of the sugar moiety results in an enzymatically stable compound [23] but this causes a strong reduction in its pEC_{50} from 8.10 (UTP) to 6.62 (UTPγS) at $P2Y_2$ receptor [14;15]. Accordingly, a complete loss of antagonist activity of UTPγS at $P2Y_{12}$ receptor is observed in the present study. Addition of an alkyl group at 5-position of the pyrimidine base resulted in reduction in its agonist activity at $P2Y_2$ receptor [14]. This phenomenon was also observed in the present study and addition of an amino-alkyl group at 5-position resulted in complete loss in its anti-platelet aggregation activity. Thiol-modification of positions 2 and 4 of pyrimidine ring in UTP possibly results in enhanced interaction of the nucleotide with the cysteine residues of $P2Y_{12}$ receptor resulting in enhanced antagonistic activity. This assumption is based on the previous studies demonstrating active metabolites of both clopidogrel and prasugrel interact with the extracellular cysteine residues of $P2Y_{12}$ receptor and is one the mechanisms of their receptor antagonism [24;25].

4. Materials and Methods

4.1 Materials: 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); uridine triphosphate (UTP), UTPγS, and ARL 67156 were from Sigma (Steinheim, Germany); Adenosine diphosphate (ADP) was from Enzo Life Science (Lörrach, 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. 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 before taking the blood after approval from the local ethics committee of University of Giessen. 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 get the 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. Platelet count in PRP was adjusted to 250-280 x 10^6 /ml by diluting native PRP with the subject's PPP.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 8 November 2016 doi:10.20944/preprints201611.0048.v1

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 %) followed by re-centrifugation at 600 x *g* for 10 min. Finally, the washed platelets were re-suspended in Tyrode's buffer (pH 7.2) at the concentration of 3 x 10^8 /ml. The suspended platelets showed 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. Indicated concentrations of agonists and/or antagonists were added in a total volume of 50 µl NaCl (0.9%) solution making final volume 500 µl. Relative platelet aggregation response was determined by comparison of light transmission through PPP (500 µl) and is expressed as percentage response. In case of ADP-induced platelet aggregation 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 binding of [^{3}H]-ADP (PerkinElmer) to platelet $P2Y_{12}$ receptors according to 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 saturated concentrations of cold ADP (1 mM). For competitive binding of UTP analogues with $P2Y_{12}$ receptors, the analogues were added at concentrations 5-fold of their respective IC_{50} values to attain complete receptor saturation. The [^{3}H]-ADP radioactivity was measured using 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 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 followed the protocol outlined by the manufacturer. The cAMP content of each well was determined using ''Infinite® 200'' multi-plate reader (Tecan, Männedorf, Switzerland). Levels of cAMP were normalized to platelet count used for the measurements.

 doi:10.20944/preprints201611.0048.v1

4.6 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, SC, 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 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 [12;14]. Here we describe for the first time that the thio-derivatives of UTP act as potent antagonists for platelet $P2Y_{12}$ receptors. Addition of thiol group at the 4-position of the uracil moiety results in highly potent $P2Y_{12}$ receptor antagonist. Future studies directing modifications of this uracil position may offer more potent $P2Y_{12}$ receptor antagonists which may be employed clinically to reduce thrombotic events.

Acknowledgements:

All experiments were performed at the Department of Cardiology and Angiology, University Hospital, Justus Liebig University, Giessen, Germany. The study was supported by Anschubfinanzierung grants to D. Gündüz and M. Aslam. The technical support by S. Schäfer, D. Reitz, and H. Thomas is gratefully acknowledged.

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

Reference List

1 Mehta, P.; Mehta, J.: Platelet function studies in coronary artery disease. V. Evidence for enhanced platelet microthrombus formation activity in acute myocardial infarction. *Am J Cardiol* 1979, *43*, 757-760.

2 Mehta, P.; Mehta, J.; Pepine, C.J.; Miale, T.D.; Burger, C.: Platelet aggregation across the myocardial vascular bed in man: I. Normal versus diseased coronary arteries. *Thromb Res* 1979, *14*, 423-432.

3 Gawaz, M.; Langer, H.; May, A.E.: Platelets in inflammation and atherogenesis. *J Clin Invest* 2005, *115*, 3378-3384.

4 Hagedorn, I.; Vogtle, T.; Nieswandt, B.: Arterial thrombus formation. Novel mechanisms and targets. *Hamostaseologie* 2010, *30*, 127-135.

5 Leon, C.; Hechler, B.; Freund, M.; Eckly, A.; Vial, C.; Ohlmann, P.; Dierich, A.; LeMeur, M.; Cazenave, J.P.; Gachet, C.: Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest* 1999, *104*, 1731-1737.

6 Storey, R.F.; Sanderson, H.M.; White, A.E.; May, J.A.; Cameron, K.E.; Heptinstall, S.: The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. *Br J Haematol* 2000, *110*, 925-934.

7 Kunapuli, S.P.; Dorsam, R.T.; Kim, S.; Quinton, T.M.: Platelet purinergic receptors. *Curr Opin Pharmacol* 2003, *3*, 175-180.

8 Hechler, B.; Gachet, C.: P2 receptors and platelet function. *Purinergic Signal* 2011, *7*, 293-303.

9 Jin, J.; Kunapuli, S.P.: Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation. *Proc Natl Acad Sci U S A* 1998, *95*, 8070-8074.

10 Kahner, B.N.; Shankar, H.; Murugappan, S.; Prasad, G.L.; Kunapuli, S.P.: Nucleotide receptor signaling in platelets. *J Thromb Haemost* 2006, *4*, 2317-2326.

11 Hollopeter, G.; Jantzen, H.M.; Vincent, D.; Li, G.; England, L.; Ramakrishnan, V.; Yang, R.B.; Nurden, P.; Nurden, A.; Julius, D.; Conley, P.B.: Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 2001, *409*, 202-207.

12 Kim, H.S.; Ravi, R.G.; Marquez, V.E.; Maddileti, S.; Wihlborg, A.K.; Erlinge, D.; Malmsjo, M.; Boyer, J.L.; Harden, T.K.; Jacobson, K.A.: Methanocarba modification of uracil and adenine nucleotides: high potency of Northern ring conformation at P2Y1, P2Y2, P2Y4, and P2Y11 but not P2Y6 receptors. *J Med Chem* 2002, *45*, 208-218.

13 Lazarowski, E.R.; Watt, W.C.; Stutts, M.J.; Boucher, R.C.; Harden, T.K.: Pharmacological selectivity of the cloned human P2U-purinoceptor: potent activation by diadenosine tetraphosphate. *Br J Pharmacol* 1995, *116*, 1619-1627.

14 Brunschweiger, A.; Muller, C.E.: P2 receptors activated by uracil nucleotides--an update. *Curr Med Chem* 2006, *13*, 289-312.

15 Jacobson, K.A.; Ivanov, A.A.; de, C.S.; Harden, T.K.; Ko, H.: Development of selective agonists and antagonists of P2Y receptors. *Purinergic Signal* 2009, *5*, 75-89.

16 Jacobson, K.A.; Boeynaems, J.M.: P2Y nucleotide receptors: promise of therapeutic applications. *Drug Discov Today* 2010, *15*, 570-578.

17 Hechler, B.; Vigne, P.; Leon, C.; Breittmayer, J.P.; Gachet, C.; Frelin, C.: ATP derivatives are antagonists of the P2Y1 receptor: similarities to the platelet ADP receptor. *Mol Pharmacol*

1998, *53*, 727-733.

18 Kauffenstein, G.; Hechler, B.; Cazenave, J.P.; Gachet, C.: Adenine triphosphate nucleotides are antagonists at the P2Y receptor. *J Thromb Haemost* 2004, *2*, 1980-1988.

19 Jacobson, K.A.; Jarvis, M.F.; Williams, M.: Purine and pyrimidine (P2) receptors as drug targets. *J Med Chem* 2002, *45*, 4057-4093.

20 Kim, H.S.; Ravi, R.G.; Marquez, V.E.; Maddileti, S.; Wihlborg, A.K.; Erlinge, D.; Malmsjo, M.; Boyer, J.L.; Harden, T.K.; Jacobson, K.A.: Methanocarba modification of uracil and adenine nucleotides: high potency of Northern ring conformation at P2Y1, P2Y2, P2Y4, and P2Y11 but not P2Y6 receptors. *J Med Chem* 2002, *45*, 208-218.

21 Storey, R.F.: The P2Y12 receptor as a therapeutic target in cardiovascular disease. *Platelets* 2001, *12*, 197-209.

22 Herbert, J.M.; Savi, P.: P2Y12, a new platelet ADP receptor, target of clopidogrel. *Semin Vasc Med* 2003, *3*, 113-122.

23 Lazarowski, E.R.; Watt, W.C.; Stutts, M.J.; Brown, H.A.; Boucher, R.C.; Harden, T.K.: Enzymatic synthesis of UTP gamma S, a potent hydrolysis resistant agonist of P2U-purinoceptors. *Br J Pharmacol* 1996, *117*, 203-209.

24 Ding, Z.; Kim, S.; Dorsam, R.T.; Jin, J.; Kunapuli, S.P.: Inactivation of the human P2Y12 receptor by thiol reagents requires interaction with both extracellular cysteine residues, Cys17 and Cys270. *Blood* 2003, *101*, 3908-3914.

25 Algaier, I.; Jakubowski, J.A.; Asai, F.; Von, K., I: Interaction of the active metabolite of prasugrel, R-138727, with cysteine 97 and cysteine 175 of the human P2Y12 receptor. *J Thromb Haemost* 2008, *6*, 1908-1914.