

# LUND UNIVERSITY

#### Novel Agonists and Antagonists of Platelet Receptors

Högberg, Carl

2011

#### Link to publication

Citation for published version (APA):

Högberg, C. (2011). *Novel Agonists and Antagonists of Platelet Receptors*. [Doctoral Thesis (compilation), Cardiology]. Department of Cardiology, Clinical sciences, Lund University.

Total number of authors:

#### General rights

Unless other specific re-use rights are stated the following general rights apply:

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights. • Users may download and print one copy of any publication from the public portal for the purpose of private study

- or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
  You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: https://creativecommons.org/licenses/

#### Take down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

**PO Box 117** 221 00 Lund +46 46-222 00 00

# NOVEL AGONISTS AND ANTAGONISTS OF PLATELET RECEPTORS



# LUND UNIVERSITY Faculty of Medicine

Carl Högberg

**DEPARTMENT OF CARDIOLOGY** 

**CLINICAL SCIENCES, LUND** 

LUND UNIVERSITY, SWEDEN

2011

Cover illustration: Docking of a farnesyl pyrophosphate (FPP) molecule (yellow and red) in the  $P2Y_{12}$ -receptors ligand binding site.

Lund University, Faculty of Medicine Copyright © Carl Högberg Tryck Media-Tryck, Lund 2011 ISBN 978-91-86671-53-2 ISSN 1652-8220 Lund University, Faculty of Medicine Doctoral Dissertation Series 2011:3

Yesterday is history. Tomorrow is a mystery. Today is a gift. That's why it is called the present.

-Master Oogway

*This thesis is dedicated to Karin, my loving wife.* 

#### Abstract

Platelets are central for the primary haemostatis. The platelet has surface bound receptors that are vital for the platelet activation. One of these receptors, the purinergic ADP-activated G-protein coupled receptor (GPCR)  $P2Y_{12}$  is a key receptor for platelet activation, and thus for thrombus formation, which makes  $P2Y_{12}$  a target in pharmaceutical antiplatelet therapies. As a complement during revascularization surgery, mild hypothermia has been accepted, but its effect on platelet activation is unclear. We performed a comparative ex vivo study on platelets, treated with mild hypothermia and normothermia in the presence of  $P2Y_{12}$ inhibitors. We found that mild hypothermia increased platelet  $P2Y_{12}$ activation and that the  $P2Y_{12}$  inhibitor, clopidogrel, lost in efficiency. The  $P2Y_{12}$  receptor is also present on vascular smooth muscle cells (VSMC), promoting contractions. We initiated a comparative study on human and murine artery tissue to evaluate if any of the antiplatelet substances, clopidogrel and ticagrelor (AZD6140) a reversibly non-prodrug P2Y<sub>12</sub> inhibitor, could inhibit P2Y<sub>12</sub> signaling also on VSMC. The results showed that ticagrelor was able to inhibit  $P2Y_{12}$  stimulated vasoconstriction, while clopidogrel had no effect. The succinate GPCR (SUCNR1) mRNA has been found at high levels in platelets. We performed a study on SUCNR1 to investigate its function in platelets. The study showed that the succinate receptor was expressed as a membrane bound Gai-protein. Succinate per se could mediate platelet activation. It was dependent on  $P2Y_{12}$  activation, tromboxane  $A_2$  release and PI3K $\beta$  signaling. By screening intermediates in the mevalonate pathway, i.e. the cholesterol synthesis, we found that one of them, farnesyl pyrophosphate (FPP), had an antagonistic effect on ADP induced platelet activation. Further investigation on FPP with [<sup>35</sup>S]GTP<sub>Y</sub>S binding and docking studies confirmed that FPP is an endogenous antagonist on the platelet  $P2Y_{12}$  receptor.

Keywords: Platelets, P2Y<sub>12</sub>, SUCNR1, hypothermia, FPP

### List of publications

This thesis is based on four studies, referred to in the text by their Roman numerals. Papers are appended at the end of the thesis.

- I. Carl Högberg, David Erlinge and Oscar Ö Braun Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment *Thrombosis Journal 2009; 7:2*
- II. Carl Högberg, Helen Svensson, Ronny Gustafsson; Atli Eyjolfsson, and David Erlinge
   The reversible oral P2Y<sub>12</sub> antagonist AZD6140 inhibits
   ADP-induced contractions in murine and human vasculature
   *International Journal of Cardiology 2010, vol 142 pp 187-192*
- III. Carl Högberg, Olof Gidlöf, Chanyuan Tan, Siv Svensson, Jenny Nilsson-Öhman, David Erlinge and Björn Olde Succinate independently stimulates full platelet activation via cAMP and PI3β kinase signaling Journal of Thrombosis and Haemostasis (in press)
- IV. Carl Högberg, Olof Gidlöf, Francesca Deflorian, Kenneth A. Jacobson, Björn Olde and David Erlinge Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y<sub>12</sub> receptor mediated platelet aggregation (*Manuscript*)

## Contents

Abstract	. 5	
List of publications6		
Contents	.7	
Abbrevations	.9	
Introduction	10	
Atherosclerosis, initiation and pathophysiology	10	
Pharmachologocal approaches to inhibit primary and		
secondary haemostasis	12	
Platelet physiology	13	
Initiated platelet activation and subsequent deactivation	14	
General classification, structure and regulation of purinergic receptors: Ionic channel receptors and G-protein coupled receptors (GPCR) on platelets	; 16	
Purinergic platelet GPCR and their agonists	18	
Synthetic antagonists for the purinergic receptors P2Y <sub>1</sub> and P2Y <sub>12</sub> Succinate and its receptor SUCNR1	18 20 20	
Aims	23	
Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment (Paper I).	23	
The reversible oral P2Y <sub>12</sub> antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature (Paper II)2	24	
Succinate independently stimulates full platelet activation via cAMP and PI3β kinase signaling (Paper III)2	24	
Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y <sub>12</sub> receptor mediated platelet aggregation (Paper IV)2	25	

Methods	6	
Myograph experiments		
Light transmission aggregometry		
Real Time-PCR (RT-PCR)2	27	
Flow cytometry2	28	
Calciumflux analysis2	29	
[ <sup>35</sup> S]GTPγS binding3	30	
Results and discussion	31	
Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment (Paper I)	1	
The reversible oral P2Y <sub>12</sub> antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature (PaperII)	4	
Succinate independently stimulates full platelet activation via cAMP and Pl3β kinase signaling (Paper III)3	7	
Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y <sub>12</sub> receptor mediated platelet aggregation (Paper IV)4	.1	
General discussion44		
Main Conclusions4	6	
Populärvetenskaplig sammanfattning4	17	
Acknowledgements		
References		
Appendix: Original Papers I-IV5	57	

## Abbrevations

ADP	adenosine 5'-diphosphate
2-MeSADP	2'-methylsulfa adenosine 5'-diphosphate
ASA	ascorbic acid
ATP	adenosine 5'-triphosphate
cAMP	adenosine 3',5'-monophosphate
СНО	chinese hamster ovary cells
COX-1	cyclooxygenase-1
DAG	diacylglycerol
FPP	farnesyl pyrophosphate
GDP	guanine 5'-diphosphate
GPIIb/IIIa	glycoprotein IIb/IIIa
GPCR	G-protein coupled receptor
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
IP <sub>3</sub>	inositol 1', 4', 5'-triphosphate
LTA	light transmission aggregometry
MI	myocardial infarction
NE	norepinephrine
NO	nitric oxide
OCS	open canalicular system
PAC-1	procaspase activating compound-1
PCI	percutaneous coronary intervention
PDGF	platelet derived growth factor
PGI <sub>2</sub>	prostacyclin I <sub>2</sub>
PIP <sub>2</sub>	phosphatidylinositol 4', 5'-phosphate
PLC	phosphlipase C
PPP	platelet poor plasma
PRP	platelet rich plasma
P-selectin	platelet selectin
5-TH	serotonin
TXA <sub>2</sub>	thromboxane A <sub>2</sub>
VASP	vasodilatator-stimulated phosphoprotein
VSMC	vascular smooth muscle cells
vWf	von Willebrand factor

#### Introduction

Cardiovascular disease is the main cause of death in the developed world [1]. Myocardial infarction and stroke are caused by haemostatic complications, in which the blood platelet has an essential role [2, 3]. Atherosclerosis is a chronic long-term disease which originates during late childhood and progresses all throughout life [4]. It is caused by a number of synergistically acting variables that effectively promotes the accumulation of inflammatory atherosclerotic plaque (atheromas). The two most important factors are lifestyle (physiological and psychosocial) and genetic factors. Variables related to lifestyle are smoking, physical inactivity and high intake of calorie rich food (fat and carbohydrates) leading to diabetes mellitus and the metabolic syndrome [5]. Factors related to the psychosocial negative lifestyle are stress and severe forms of psychosocial illness, i.e. depression [6]. Other inherited important risk factors are: gender, genetic polymorphisms, type 1-diabetes, hypertension and age [7].

#### Atherosclerosis, initiation and pathophysiology

Atheromas are initiated by migration of low density lipids (LDL), through the endothelial cell (EC) barrier into the extracellular matrix (ECM) of the intima [8]. The cholesterol rich LDL-particles collected in the intima, becomes oxidized and initiate inflammation by promoting local release of chemotactic cytokines, particularly macrophage chemoattractant protein -1 (MCP-1). Macrophages, recruited by MCP-1, will accumulate in the intima matrix where they will phagocyte the oxidized LDL-particles [9]. Doing so, the macrophages change their chemical constitution and morphology ending up as so called foam cells. The foam cells are not able to process the oxidized-LDL, and eventually, they will enter apoptosis depositing a greater amount of oxidized cholesterol into the artery wall. The macrophage migration and turnover into foam cells is the origin of the lipid rich core of the atheroma. Platelet recruitment occurs in the parts of the artery where atheromas rupture and the EC layer is damaged [10]. Platelets release mediators, e.g. platelet derived growth factor (PDGF), which promotes migration

of vascular smooth muscle cells (VSMC) into the intima, and also inflammatory ligands, for example CD154, Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES) which regulates activation of CD40 which, in turn, promotes expression of pro-atherogenic mediators [11]. Platelet also release Transforming Growth Factor beta (TGF- $\beta$ ) which promotes collagen I production in the ECM [12]. Collagen production in the ECM is stimulated by recruitment of VSMC into the intima [13]. Artherosclerotic plaque can, depending on their ability to promote thrombus formation, be categorized as "stable" or "vulnerable". If the plaque contains a fibrous cap of VSMC that safely withhold the lipid core from the lumen, the plaque is characterized as a "stable" one. If, on the other hand the plaque has a large lipid core with a poor developed fibrous cap, the plaque is considered to be "vulnerable" [14] (Fig 1).



**Fig 1.** Left: Illustrating a "stable" plaque. Centre: Illustrating a "vulnerable" plaque. Right: Illustrating a vulnerable eruptive plaque which initiate a thrombus formation and thus, vascular occlusion which in turn can onset angina pectoris, stroke or a MI.

If a plaque ruptures, collagen and vWf are exposed and will rapidly adhere and recruit platelets, creating large thrombi. The thrombi can initiate a massive coronary occlusion subsequently leading to a stroke or an acute MI, causing a massive necrosis of the myocardium with a possible mortal outcome [15]. To save a patient with MI the coronary vessels must be revascularized, either pharmacologically through fibrinolysis [16] or mechanical through percutaneous coronary artery is expanded by balloon angioplasty and stenting. However, the metal stent may stimulate platelet aggregation and cause in-stent thrombosis [18]. Patients receiving a metal stent must take a dual antiplatelet therapy ascorbic acid

(ASA) and an ADP-receptor antagonist [17]. If the patient suffers from multivessel disease, Coronary Artery Bypass Graft (CABG) surgery can to be performed where an artery or a vein is used to bypass the stenotic area [17].

# Pharmacological approaches to inhibit primary and secondary haemostasis

Patient suffering from coronary heart disease are treated with several medications to prevent progression of the disease and the development of myocardial infarction. The therapy is usually aimed at inhibition of both the primary (platelets) and secondary phase (coagulation) in haemostasis. Platelet inhibition is achieved through direct inhibition of enzymes or receptors, such as the thromboxane A<sub>2</sub> (TXA<sub>2</sub>) generating enzyme, (COX-1) using ASA, or the ADP receptor  $P2Y_{12}$ , present on the platelet surface. Another target is the glycoprotein (GP) IIb/IIIa integrin also situated on the platelet surface. GPIIb/IIIa is a membrane-protein with a central role in the platelet-platelet aggregation but also in the initiating adhesion stage to vWf in ECM [19]. Common inhibitors of the fibrin generation phase are warfarin, heparin, fractionated heparin (LMWH), thrombininhibitors (bivalirudin) or Xa-inhibitors (fondaparinux) [16, 19]. Other useful substances in the treatment of coronary heart disease are Nitroglycerine which promotes dilatation of blood vessels and Beta blocking agents (metoprolol, propranolol) that inhibits  $\beta$ -receptor activity, thus reducing heart rate and blood pressure [20]. Therapies aimed at lowering the cholesterol levels are often used as a way of controlling the development of atherosclerosis. Cholesterol reducing compounds such as simvastatin, atorvastatin and rosuvastatin belong to a group called stating which function by inhibiting the rate limiting enzyme in the mevalonate pathway, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase) [21].

#### Platelet physiology

The platelets or thrombocytes (after the Greek word thrombos - clot and cytos - cell) have a central role in the haemostatic machinery. Platelets main function is to stop vascular hemorrhage, but platelets also contribute by increasing the inflammatory and atherosclerotic process. Once activated, from a resting state, the platelets work in concert with the cross linked fibrin network to repair liaisons in the EC barrier of the vascular wall lumen.

The platelets circulate in the blood plasma, where they are present in high concentrations (150 - 400,000 cells/ $\mu$ L in whole blood) [22]. Platelets are created through endocytosis from larger polynuclear cells called megakaryocytes [23] and have a life span of approximately 7-10 days. The megakaryocytes are present in the bone marrow matrix and produce 5,000 to 10,000 platelets per day [23]. Old platelets are removed either by macrophages, through phagocytosis, or by the liver and spleen.

The resting platelet is discoid with the dimension of 3 times 0.5µm in width, respectively [22]. The platelet is an anuclear cell, but have retained many cell structures, e.g. plasma membrane, mitochondria, dense tubular system (store of  $[Ca^{2+}]_i$ , and occasional Golgi elements and ribosomes (Fig 2). Platelets also contain membrane-bound granules that, depending on type, have different composition of active substances. The alpha-granules contain several proteins i.e. growth factors like platelet derived growth factor (PDGF) important for endothelial activation, coagulation proteins like thromboglobulin, fibronectin and fibrinogen. The alpha granules contain adhesion factors, as vWF and GPIIb/IIIa, and they hold platelet specific proteins, e.g. coagulation factor V (which activates thrombin) and CD62P (P-selectin) [24]. P-selectin is an important protein that facilitates platelet-platelet and platelet-leukocyte binding. Platelets also contain delta-granules, sometimes referred to as dense opaque core because of their elevated electron density when observed through an electron microscope. The delta granules holds high concentration of small molecules, for instance ATP, ADP pyrophosphate (P<sub>i</sub>), 5-HT and ions like  $Ca^{2+}$  and  $Mg^{2+}$  [24]. These small molecules are vital for the platelets further activation and recruitment of other platelets.



Fig 2. Illustrative figure describing the physiology of a resting platelet.

# Initiated platelet activation and subsequent deactivation

Platelet activation is initiated when the disrupted endotelial cell layer exposes collagen and vWf, that is present in the subendotelial intima [25]. Collagen is an important part of the connective tissue and also one of the most expressed proteins in the intima [25]. The platelet activation starts initially by rolling and tethering platelets on the surface of the exposed subendotelial area. The platelet GPIb-IX-V integrin complex connects to exposed collagen-bound vFf. This initial adhesion stage becomes stabilized through  $\alpha_2\beta_1$  integrin-collagen interaction and further GPIIb/IIIa-vWf attachment [25, 26]. The stable adhesion enables a conformational shape change of the platelet which irreversibly alters the cell platelet morphology. The components that reorganizes the cytoskeleton, the sub-membrane microtubulin and the cytoplasmic actin filaments, alters the cell structure of the

platelet, and the once discoid shape becomes spherical. The spherical shape then flattens and lamellae begin to stretch out from the inner core of the platelet and the organelles and granules get squeezed into the center of the activated platelet [27]. The alpha and delta granules are fused by exocytosis through an open canalicular system (OCS). Molecules released from the platelet vesicles, ADP, P-selectin and fibrinogen, now interacts with membrane bound receptors on other cell types present in the vascular lumen, e.g. leukocytes, endothelial cells and other platelets. The meshwork of platelets and fibrin grows in the ulcerated plaque, which might end up in forming a thrombus-generated occlusion. These processes are inhibited by the vessel wall endothelium which releases nitric oxide (NO) and prostacyclin  $I_2$  (PGI<sub>2</sub>) into the lumen and thereby stop and de-activate the thrombus formation [28] (Fig 3). This suppression of the platelet is simultaneously followed by release of endogenous plasmin. Plasmin is generated from thrombin-induced cleavage of plasminogen into plasmin, which lyses the fibrin cross linked meshwork and disperse the thrombus [29].



Fig 3. Illustrating platelet activation in artery lumen where a vascular lesion exposes the collagen rich subendothelial matrix. The circulating resting platelets activates by collagen and surface expressed vWf. During total activation the platelets release their granular content which recruits and activates new platelets to the damaged area. The surrounding endothelium releases anti-thrombotic mediators as nitric oxide (NO) and prostacyclin (PGI<sub>2</sub>) to inhibit further thrombus formation.

### General classification, structure and regulation of purinergic receptors: lonic channel receptors and G-protein coupled receptors (GPCR) on platelets

The purinergic receptor present on the platelet is either an ionic channel receptor or a G-protein coupled receptor (GPCR). The ionic channel receptor P2X<sub>1</sub> is a transmembrane protein that becomes activated upon ATP binding to its extracellular binding site [30]. The activated receptor protein opens an inward rectifying ionic gate whereby ionic-currents of Ca<sup>2+</sup> leaks in and promotes an initiation of platelet shape change The P2X<sub>1</sub> is an oligomer that is build up by three subunits where every subunit contain two transmembrane (TM)  $\alpha$ -helixes (Fig 4). The purinergic GPCR is also activated by mediators from the extracellular side, initiating an intracellular G-protein coupled signaling cascade. In the platelet, these GPCR activated transduction cascades often lead to massive platelet activation resulting in shape change, aggregation and vesicular release.

A GPCR is also known as a seven transmembrane receptor (7TM). The general structure of a GPCR is characterized by a single protein chain with an extracellular N-terminal followed by 7TM  $\alpha$ -helices connected by three extracellular and three intracellular loops and an intracellular C-terminal tail. The tertiary structure of a GPCR resembles a barrel with the 7TM helices surrounding a central cavity forming the ligand binding domain (Fig 4). The intracellular side of the GPCR is normally bound to a heterotrimeric G-protein composed of a G $\alpha$ -GTP subunit and a G $\beta\gamma$ -subunit. Once the GPCR is activated, by binding of an agonist, the receptor shifts conformation thus activating the G-protein causing it to detach from the receptor. The activated G $\alpha$ -subunit releases guanine 5'-diphosphate nucleotide (GDP) from the binding pocket and exchanges it into a guanine 5'-triphosphate nucleotide (GTP). This exchange promotes separation of the  $G\beta\gamma$ -subunit from the  $G\alpha$ -subunit. Both the  $G\alpha$ - and the  $G\beta\gamma$ -subunit promote or inhibits enzymatic activity by interacting with key enzymes. Depending on the type of  $G\alpha$ -subunit different second messengers systems will be activated. For example, acivation of Gas-subunits will stimulate adenylate cyclase (AC) and cAMP production while Gai-subunit will inhibit AC, causing a decrease in the cytoplasmic levels of cAMP.



*Fig 4.* Illustration of structures of a typical ATP activatied ion channel receptor like the  $P2X_1$  and a classic GPCR like the  $P2Y_1$  or  $P2Y_{12}$  receptor.

#### Purinergic platelet GPCR and their agonists

The main purinoreceptors responsible for platelet activation are the  $P2Y_1$  and  $P2Y_{12}$  receptors. The primary agonist for both these receptors is the adenine nucleotide adenosine 5'-diphosphate (ADP) (Fig 7). The P2Y<sub>1</sub> receptor is a Gaqcoupled receptor. Gaq-proteins regulate the activation of the intracellular Phospholipase (PLC) which hydrolyzes the membranebound C phosphatidylinositol 4', 5'-phosphate (PIP<sub>2</sub>) into diacylglycerol (DAG) and the second messenger molecule inositol 1', 4', 5'-triphosphate (IP<sub>3</sub>) [30]. Released IP<sub>3</sub> activate IP<sub>3</sub> receptors present on the membrane of the calcium stores in the platelets dense tubular system [31, 32] and trigger calcium release. This calcium flux initiate the reorganization of the cytoskeleton and thus the platelet shape change [32].  $P2Y_1$  is a low affinity receptor and is not so highly expressed on platelets as  $P2Y_{12}$  [33]. The high affinity  $P2Y_{12}$  receptor is a Gai-coupled receptor which inhibits adenylate cyclase resulting in a reduction of the intracellular signaling messenger cAMP (Fig 7), while the G<sub>βγ</sub>-subunit activates an enzyme known as phosphatidylinositol-3-kinase (PI3K). PI3K promotes the phosphorylation and activation of the downstream effector enzyme Protein Kinase B (PKB) also known as Akt1, a protein that has been reported to play a role in optimal activation of GPIIb/IIIa [34].

# Synthetic antagonists for the purinergic receptors $P2Y_1$ and $P2Y_{12}$

Two of the most frequently used synthetic  $P2Y_1$  antagonists are MRS2179 and MRS2500. These substances are both competitive antagonists that bind reversibly to the active site of the receptor and thus inhibit receptor activation [35, 36] Half maximal inhibiting concentration values (IC<sub>50</sub>) are 0.23µM [35] and 1nM [36] for MRS2179 and MRS2500, respectively.

A clinically used antagonist for the  $P2Y_{12}$  receptor is clopidogrel (Fig 5). Clopidogrel, distributed as  $Plavix^{(B)}$  is together with ASA (ascorbic acid) used as standard antithrombotic treatment in coronary heart disease and as follow up treatment after PCI to reduce post-stent thrombotic complications [37, 38]. Clopidogrel is a prodrug, and needs to be converted by the liver enzyme cytochrome P450 (CYP450) into its functionally active metabolic form [38]. The

active metabolite of clopidogrel is an irreversible ligand that binds covalently to the binding site, blocking the receptor for interaction with native agonists [39]. Clopidogrel resistance, caused by individual differences in the enzymatic conversion capacity of CYP450, has become a serious problem in anti-thrombotic treatment [40, 41]. As much as 50 percent of the patients are non- or poor responders to clopidogrel [41]. Another problem is that the irreversible binding of this compound can complicate acute surgical operations. This has called for the development of new precise, stable antithrombotic drugs with less variability in effect. Amongst them is the substance cyclopentyl-triazolopyrimidine, ticagrelor (AZD6140) (Fig 5). Ticagrelor is not a prodrug and it binds reversibly to the P2Y<sub>12</sub> receptor and can also reach high plasma concentrations, which makes it a possible antagonist for P2Y<sub>12</sub> receptors present on VSMC, preventing ADP mediated artery contractions [39, 42]. Ticagrelor has also the benefit in fast plasma clearance and thus enables acute interventions in patients. Clinical studies as the PLATelet inhibition and patient Outcome (PLATO) 2010 [43] confirmed the results from the earlier Dose confirmation Study assessing anti Platelet Effects of AZD6140 vs. clopidogRel in non-ST segment Elevation myocardial infarction -2(DISPERSE-2) study[44] where the advantages of ticagrelor vs. clopidogrel, as antiplatelet therapy in patients suffering from acute coronary syndromes, were evaluated and confirmed.



*Fig 5. Chemical structures of synthetic P2Y*<sub>12</sub> *receptor antagonists.* 

#### Succinate and its receptor SUCNR1

The succinate receptor number 1 (SUCNR1), is a GPCR. SUCNR1 is expressed in most body organs with high expression in liver and kidneys [45]. SUCNR1 is a protein composed of a 330 amino acids. The SUCNR1 is a low affinity receptor needing high agonist concentrations to become activated. The key ligand for the SUCNR1 is succinate [46] (Fig 7). Succinate is a physiological important compound generally known as an intracellular intermediate in the citric acid cycle [47]. Levels of succinate present in the plasma are approximately 20µM [48, 49]. During pathophysiological conditions like ischemia and metabolic stress, succinate plasma levels have been reported to increase and reach millimolar concentrations [49-52]. Succinate has also in a recent rapport been identified to be a paracrine signal molecule released upon high levels of glucose, induces SUCNR1stimulating renin release from kidneys and activates the renin-angiotensin system, thus leading to an increase in blood pressure.[49]. However, succinate has previously been reported to have an potentiating effect in ADP and epinephrine induced platelet activation [53], but the succinate receptor and mechanism of action was unknown until SUCNR1 were characterized on the platelet surface [46].

#### Farnesyl pyrophosphate (FPP)

The isoprenoid farnesyl pyrophosphate (FPP) (Fig 7) is an important intermediate in the mevalonate pathway (Fig 6). The mevolonate pathway is central for the cholesterol synthesis mainly taking place in the liver and intestines [54]. FPP is identified as a precursor molecule for the synthesis of haem A, ubiquinone (important mediators in the electron transport chain) and dolichol, which functions as a membrane anchor for the formation of oligosaccharides [54]. FPP is further involved in farnesylation of several proteins (e.g., Ras, Rho, Rac) that modulate a variety of cellular processes including signaling, differentiation, and proliferation [55]. In the farnesylation process, Farnesyl Transferase (FTas) catalyzes the transfer of the non-polar 15-carbon backbone of FPP to the protein thus enabling the protein to attach to the hydrophobic membrane structure where it later becomes activated [56]. The small intracellular protein GTPase Ras functions as downstream effectors for gene transcription and rapid cell proliferation. Ras have been characterized as a protein involved in the development of several malign forms of cancer [55, 56], identify FTas as target protein in drug development [57]. FPP has lately been assigned a new role, namely as a potential agonist. Free FPP has been reported to function as an agonist for the Lysophosphatic acid GPCR LPA<sub>5</sub> (GPR92) having potentiating effects on LPA-mediated shape and change. This we could confirm in our studies [58]. Recent work has also identified FPP as a potent agonist on the ion channel receptor, the Temperature-sensitive transient receptor (TRPV1) [59].



*Fig 6.* Schematic view over the mevalonate pathway. *FPP* (in red square) is a central mediator in the mevalonate pathway.



Fig 7. Chemical structures for agonists and secondary messengers.

### Aims

The aim of this thesis was to examine different aspects of the platelet activation, except in paper II where focus was on how an important platelet receptor  $(P2Y_{12})$  activates smooth muscle cells in the vascular wall. Furthermore, the thesis concerns different aspects of platelet GPCR activation; with a central aspect on the purinergic ADP activated P2Y<sub>12</sub> receptor.

#### Mild hypothermia does not attenuate platelet aggregation and may even increase ADPstimulated platelet aggregation after clopidogrel treatment (Paper I)

Mild hypothermia is used as a recommended standard treatment for patients treated for a cardiac arrest [60]. Although several mechanisms probably are involved, the general hypothesis is that by cooling the body core temperature the metabolism becomes depressed whereby damaged tissue recovers easier. Clinical studies have reported beneficial effects of hypothermia with reduced mortality and improved neurological outcome in unconscious patients affected by cardiac arrest [61-64]. Hypothermia could also have effects on the haemostatic system. Coagulation is impaired since the activity of involved coagulation enzymes is reduced. For platelets it has been reported both reduced [65, 66] and increased activity [67-70]. Most patients with cardiac arrest are in need of anti-thrombotic treatment, because myocardial infarction is often the primary cause of the disease. It is therefore important to know how hypothermia affects platelet function and platelet inhibiting drugs.

From this background we initiated a study named "Cool Platelets" This study had its origin from a clinical perspective and aimed to investigate the effect of mild hypothermia on platelet activation *ex vivo*. Moreover, a study was also performed with the  $P2Y_{12}$  blocking agents' clopidogrel and AZD6140 (ticagrelor) to

investigate if there were any differences in their ability to oppose platelet activation during hypothermic comparing to normothermic conditions.

# The reversible oral P2Y<sub>12</sub> antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature (Paper II)

The purinergic ADP activated  $P2Y_{12}$  receptor is well studied on platelets where it plays a central part in the platelet activation machinery [71]. The  $P2Y_{12}$  receptor is also identified on VSMC where it mediates contraction upon activation. Paper II aimed to investigate if antiplatelet substances with the  $P2Y_{12}$  receptor as target could inhibit  $P2Y_{12}$  receptor signalling in platelets as well as inhibiting VSMC contractions, having a dual pharmacological therapeutic effect. The study also aimed to compare the two antiplatelet drugs, clopidogrel and ticagrelor, in order to evaluate the therapeutic effect on ADP mediated vascular contractions.

# Succinate independently stimulates full platelet activation via cAMP and PI3β kinase signaling (Paper III)

The succinate receptor SUCNR1 (GPR91), belongs to the gene superfamily of GPCR, and He *et al.* [46] has in an earlier report recognized SUCNR1 as a major target for physiological succinate. Succinate was studied by Huang *et al.* 1984 [53] and was identified as a substance with a potentiating effect for known platelet agonists e.g. ADP. The SUCNR1 is expressed in many organs and tissues, e.g. in the liver, spleen, blood vessels and is most abundant in the kidneys [45]. In an earlier report, aiming at identifying all GPCR (including orphan receptors) on the platelet membrane [33], the succinate receptor was found to be the third highest expressed GPCRs of all on the platelet. With this knowledge about high transcript expression of the succinate receptor in platelets we intended to examine the function of SUCNR1s in platelets. We also aimed to evaluate to which G-protein

SUCNR1 is linked and thus, to study the intracellular pathways that the SUCNR1 stimulates.

#### Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y<sub>12</sub> receptor mediated platelet aggregation (Paper IV)

Paper IV is based on an earlier report aimed to identify all GPCRs on the platelet membrane [33]. The study recognized high expression of one of the GPCR suggested being a receptor for farnesyl pyrophosphate (FPP). FPP is an intermediate substance in the mevalonate pathway. Intermediates in the mevalonate pathway have, in earlier studies, been suggested to be involved also in functions other than the synthesis of cholesterol. These facts captured our interest. In Paper IV we performed a study aimed to evaluate if FPP had any function in platelet activation.

#### Methods

This chapter is presenting a general description of methods applied throughout the thesis.

#### Myograph experiments

A myograph is a technical device that measures and record tension from the VSMC in blood vessels. Blood vessels are cut into small segments with a few mm in width. The registered contractile force is measured in Volt, which further can be transformed into Newton - defined as the amount of net force required to accelerate a weight of one kilo (kg) at a rate of one meter per square second  $(s^2)$ . The vessels are dissected from connective tissue and further cut into vascular segments. Segments are later mounted between two holders with help of a metal thread; where one of the holders are connected to a force - displacement transducer for continuous recording of the isomeric tension. The other segmentholder allows fine adjustments of the vascular resting tension. The segments are held in temperature-controlled (37°C) tissue baths with a physiological Krebs buffer solution, pH at 7.4. The resting force (the baseline) of the vessel segments is fixed after 30 - 45 minutes. After the baseline has been set the vessel segments are stimulated with a potassium rich organic buffer which allows the VSMC to contract and reach its tension peak value. After the segment peak value is registered in the myograph the resting tension is restored through repeated washing with potassium free physiological Krebs buffer. The dilated vessel segments are allowed to rest for 25-30 minutes before an additional washing with buffer. The vessel segments are now ready to be stimulated with agonists in the presence or absence of selective antagonists.

#### Light transmission aggregometry

Light Transmission Aggregometry (LTA) is a method for *ex vivo* measurement of platelet activation in platelet rich plasma (PRP) or washed platelets, when stimulated with natural or synthetic agonists. LTA is based on turbidometry, and was first used and invented by Gustav Born (1962) [72]. In the turbidometric LTA technique the PRP is under constant stirring in a quartz cuvette through which a beam of visible light pass and the ability of the PRP block light is compared with an unstimulated control PRP. The turbidity of the PRP is proportional to the degree of aggregation and PRP becomes more transparent in the presence of activating compounds.

#### Real Time-PCR (RT-PCR)

Ouantitative RT-PCR (gRT-PCR), is a method to measure the level of mRNA in a sample. The RT-PCR methodology is based on the PCR technique where complimentary DNA (cDNA) is synthesized using isolated cellular mRNA as template. The enzyme reverse transcriptase reads the mRNA transcript and builds the complementary fragment of single stranded cDNA. The cDNA is then amplified using primers constructed to give a product of approximately 200 base pairs (bp). The gene amplification occurs in cycles where each cycle consists of three steps; Step 1 -denaturation of the dsDNA into ssDNA (95C, 1 sec); Step 2 annealing of primers to the ssDNA; Step 3 - elongation where the annealed primers are extended to a product. A fluorescent dye usually SYBR Green, with the ability to emit light when bound dsDNA is included in the reaction mixture. The principle of the quantification is simple. As the PCR product accumulates it will bind more dye and the fluorescence will increase. The result is normalized to a housekeeping gene, the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or Actin  $\beta$ , which is present in all types of cells at relatively constant levels. A convenient way to evaluate the ratio of gene expression is to use the comparative  $2^{-\Delta\Delta CT}$  method, further described by Livak and Schmittgen [73].

#### Flow cytometry

Flow cytometry is a technique where single types of cells are sorted by their physical characteristics, e.g. size or density. One of the most used methods in flow cytometry is a technique known as Fluorescence-activated cell sorting, (FACS). The FACS technique is based on specific antibodies that covalently bind to the proteins of interest and to which later secondary antibodies marked by different fluorescence dyes attach. Cells that have become marked by the fluorophore labeled proteins can be detected, sorted and isolated. The cellsorting technique is divided into several steps: 1. The cells flow in a narrow rapid stream of liquid and becomes separated relative to the diameter; 2. Cells are separated and becomes further separated into droplets with one cell per droplet; 3. Cells contained in droplets will pass through a fluorescence-monitor where a laser will excite the fluorophore-labeled cells and then isolate them through an electrostatic mechanism. The FACS technique can be used to study if a specific protein in the platelet activation machinery becomes activated in the presence of different agonists.

The Procaspase Activating Compound 1 (PAC-1) antibody is used for measuring the activation of membrane-bound GPIIb/IIIa [74]. The activation of GPIIb/IIIa is regulated through phosphorylation by an upstream affecting secondary messenger protein, the VAsodilating-Stimulated Phosphoprotein (VASP) [75]. VASP is also involved in the regulation of the cytoskeleton reorganization of the activated platelet, which initiates the platelet shape change [76]. The rate of phosphorylated (activated) and de-phosphorylated (inactivated) form of VASP can be measured using certain antibodies. The  $\alpha$ -granule exocytose, following platelet activation, releases the unique P-selectin (CD62P) protein that becomes expressed on the platelet surface and hence promote platelet-leukocyte adhesion [77]. The rate of platelet activation can be measured through the presence of P-selectin by using directed specific antibodies.

#### Calcium flux analysis

Intracellular calcium  $[Ca^{2+}]_i$  release is vital in the primary stage of platelet activation. The release of  $[Ca^{2+}]_i$  is initiated by extracellular stimulation and hence activation of a receptor linked Gag-protein [78]. The Gag-protein regulates activation of the enzyme Phospho Lipase C (PLC) which promotes hydrolysis of  $PIP_2$  into the membrane-bound DAG and the secondary messenger  $IP_3$ . The  $IP_3$ interacts with a specific IP<sub>3</sub> receptor (IP<sub>3</sub>R) situated on the membrane of the dense tubular system (DTS), which regulates the  $[Ca^{2+}]_i$  release from the calcium stores into the cytosol. The increasing concentration of calcium in the platelet causes reorganization of the cytoskeleton, i.e. platelet shape change. The intracellular calcium signal from an activated platelet in a calcium free buffer is measured through fluorescence. A calcium ion indicator, e.g. the non-fluorescent Fluo-4 acetoxymethyl ester (AM), passes the plasma membrane into the cytosol where an esterase hydrolyzes the ester vielding the fluorescent Fluo-4. As the product Fluo-4 is unable to traverse the plasma membrane, it becomes intracellular trapped. Upon combination with Ca<sup>2+</sup> the Fluo-4 becomes highly fluorescent at certain wavelengths (Fig 8).



Fig 8. Extracellular stimuli on the platelet  $G\alpha q$ -receptor initiate a mobilization of cytosolic calcium through  $IP_3$  turnover. The calcium ions activate the incorporated Fluo-4 molecule into a more excitable formation whereby excitation light becomes emitted and read spectrophotometric at 488 nm.

# $[^{35}S]GTP\gamma S$ binding

Several of the membrane bound receptors present on the platelets are members of the super family of receptor proteins, known as the G-protein coupled receptors (GPCR). The GDP is a nucleotide that is bound to the inactive form of the  $G\alpha$ subunit in a GPCR. When the agonist activates the GPCR the receptor goes through a conformation change, which ends up in the  $G\alpha$  -subunit exchanging the GDP for GTP [79]. The GTP enhances dissociation of the  $G\alpha$  -subunit from the receptor, activating the  $G\alpha$ -GTP and  $G\beta\gamma$  subunits (Fig 9). The activated subunits will in turn regulate intracellular downstream effectors. The cycle is completed by deactivation of the Ga -GTP, through the action of a GTPase, followed by reassociation of the receptor/heterotrimeric G-protien complex [79]. Exchanging the native, active GTP nucleotide for a stable isotopic labeled form, i.e.  $[^{35}S]GTP\gamma S$ , makes it possible to detect and measure the amount of activated Gi-protein. As the  $[^{35}S]$ GTP $\gamma$ S-molecule is resistant to hydrolysis by the GTPase, the accumulation of G-protein occupied by  $[^{35}S]GTP\gamma S$  in the plasma membrane upon receptor stimulation, provides a simple quantification of receptor linked Gi-protein activation [80].



**Fig 9.** Illustration of the intracellular transduction pathway in a GPCR. When the agonist docks into the GPCR binding site, it initiates a conformational change in the inward rectifying cytosolic part of the receptor, which in turn enhances a nucleotide exchange from GDP into GTP in a receptor subunit protein, defined as Ga. This nucleotide exchange triggers an uncoupling mechanism which accomplishes dissociation of the receptor/heterotrimeric (Ga and G $\beta\gamma$ ) G-protein complex. The subunit dissociation becomes the start signal for several intracellular transduction cascades that finally will modulate the platelet morphology.

#### Mild hypothermia does not attenuate platelet aggregation and may even increase ADPstimulated platelet aggregation after clopidogrel treatment (Paper I)

The strategy employed in this paper was to investigate platelets from freely healthy, non medicated free volunteers (n=8) that received a maximal dose, 600mg *per os* (p.o.) of the thiopyridine clopidogrel, trademarked as Plavix®. The volunteers donated blood samples the day before as a control. The collected PRP samples – both control and sample, were incubated at two different temperatures, 33C and 37C before LTA testing with several agonists as collagen, epinephrine, 5-TH, ADP and antagonists, MRS2500 (inhibit P2Y<sub>1</sub> signaling) and ticagrelor (inhibit P2Y<sub>12</sub> signaling).

P2Y<sub>12</sub> receptor activation inhibits the synthesis of cyclic adenosine 3', 5'monophosphate (cAMP) by blocking the ATP turnover into cAMP by adenylyl cyclase. cAMP is a regulating secondary messenger for the enzyme phosphokinase A (PKA). PKA is a regulator for the phosphorylation of a downstream effector protein known as VASP. A FACS study was performed with a specific VASP assay to investigate how mild hypothermia affects platelet activation. No significant effects could be seen between mild hypothermia compared to normothermia in the presence or absence of clopidogrel in ADP mediated inhibition of VASP phosphorylation.

Although ADP activation in platelet was not attenuated by mild hypothermia the study resulted in interesting data (Fig 10). We observed a slightly increased activity in platelets exposed to mild hypothermia compared to normothermic circumstances (Fig 11). This observation has been reported by Xavier *et al.* [69] and Hartwig *et al* [22] whereas the latter described that cold induced platelet activation by shape change mimics the agonistic response. Secondly, platelet inhibition by clopidogrel lost some of its blocking potency effect in the presence of mild hypothermia. This pattern of decreased effect was also seen in the presence of ticagrelor and by the potent P2Y<sub>1</sub>-inhibitor MRS2500. The problem with clopidogrel resistance was first reported by Järemo *et al.* [40] suggesting that

it could be beneficial to use a more potent ADP inhibitor to patients with clopidogrel resistance. In conclusion, mild hypothermia can not independently be relied on as an anti-platelet therapy and as the potency of clopidogrel is decreased by mild hypothermia. The antiplatelet therapy should not be discontinued; instead hypothermic patients may even need more potent antagonists.



**Fig 10.** LTA study on platelets, ex vivo, normothermia  $(37^\circ)$  (•) vs. mild hypothermia  $(33^\circ)$  ( $\circ$ ). (A) No difference in ADP induced platelet activation in control between 37C vs. 33C. (B): Significant difference in platelet activation was seen between 37C vs. 33C in platelets treated with clopidogrel, p.o. (C) Ticagrelor 10µM, ex vivo, lowered the platelet activation further in both 37C and 33C. No significance was seen between 37C vs. 33C. (D): When platelets treated with clopidogrel, p.o, were treated with 1µM of the P2Y<sub>1</sub> blocker MRS2500 the significance withholds and the platelet activation decreased in both 37C and 33C.



**Fig 11.** Dose-response in ADP activation on platelets in normothermia compared to mild hypothermia. A shift to the left in the diagram on platelets incubated in  $33^{\circ}C$  ( $\circ$ ) compared to  $37^{\circ}C$  ( $\bullet$ ), indicates an increased activation in platelets during conditions of mild hypothermia.

# The reversible oral P2Y<sub>12</sub> antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature (Paper II)

Both a murine *in vivo* and a human *ex vivo* model were used for the study. In the murine animal model, aorta was used to measure inhibition of 2-MeSADP induced contractility in the presence of clopidogrel and ticagrelor (AZD6140). In the human model small omental arteries and Left Interior Mammary Arteries (LIMA) were obtained during bypass surgery from 9 patients, mean age = 72 years of which 7 were men. The blood vessels were prepared and used in the same manner as vessels in the murine model. Female mice in the groups of two (5 test animals and 4 vehicles) were used in the experiment. The test animals were treated per os. twice with clopidogrel (50mg/kg). The administration of clopidogrel took place the day before and 2 hours before sacrifice of both groups of animals. Thoracic aortas were harvested through dissection. The connective tissue was removed from the vessels and they were cut into smaller segments. The aorta segments were then denuded, and the endothelial cells removed by gentle rubbing with a wooden stick.

The segments from mice aorta and human small arteries were mounted in a myograph in a 37°C controlled organ bath. The myograph settings have been described by Malmsjö *et al.* [81] and Wihlborg *et al.* [82]. After a period of rest the mice segments and small human arteries were pre-contracted to 1 mN while the LIMAs, with wider total diameter, were pre-contracted to 2 mN. Maximum contraction of segments were obtained in the presence of 60 mM K<sup>+</sup> after pre-treatment with ticagrelor and vehicle segments with 1:1000 dimethyl sulfoxide (DMSO). To study antagonistic effects of clopidogrel and ticagrelor, the blood vessels were first slightly pre-contracted with 10nM norepinephrine (NE), and then further contracted by using the stable synthetic ADP analog, 2-MeSADP. To exclude contractile response from the P2X<sub>1</sub> receptor, the P2X<sub>1</sub> receptors present in the VSMCs were all desensitized with 10µM  $\alpha$ ,β-methylene ATP ( $\alpha\beta$ -MeATP) for 10 min as described by Kasakov and Burnstock [83] (Fig 12). The results, generated by the myograph studies, are presented as percent of the K<sup>+</sup> mediated contractions.



**Fig 12.** Illustrating a representative myograph experiment, where AZD6140 (ticagrelor) where added in the treated artery segments and DMSO in equal volumes were used in the control segments. Red arrows indicate the myograph-registered blocking effect of ticagrelor on  $P2Y_{12}$ - induced vascular contractions.

The study showed a dual therapeutic action by ticagrelor with effects also on ADP-mediated contraction apart from the previously shown platelet inhibitory effects. In contrast, clopidogrel did not have any effect on vascular contractions. This was seen both in the murine and the human arteries. Murine aortas from clopidogrel in vivo treated trial animals displayed no difference in 10µM 2-MeSADP mediated contractile responses compared to vehicle animals. However 1µM ticagrelor inhibited vascular contractions in the absence and presence of clopidogrel when added ex vivo (Fig 13). Ticagrelor added ex vivo to segments from human LIMA significantly differed from control in reducing 2-MeSADP initiated contractions. This decrease in vascular contractility was also seen in the pericardial fat (small) arteries (Fig 14). The specificity of ticagrelor on the  $P2Y_{12}$ receptor were evaluated as there were no difference seen between NE induced  $\alpha_{2a}$ mediated contractions in either human LIMAs or human small artery vessels. This study is based on the observation that the  $P2Y_{12}$ -receptor is expressed on the VSMC where it promotes artery-contractions [84]. The clinical advantage with a drug that simultaneously acts on two target areas is obvious. The interpretations of the outcome of this study are that the orally administrated prodrug clopidogrel is not able to block P2Y<sub>12</sub> mediated vessel-contractions in murine vessels, even if present in high concentrations. However, ticagrelor added ex vivo displayed distinct effects on all types of vessels tested in both murine and human tissue. Ticagrelor has been shown to have effect on various cell types. The antiplatelet potential is clearly recognized through several clinical studies. This study confirms
that ticagrelor also prevents from dangerous vasospasm which is especially harmful in stenotic arteries. Also, ticagrelor could be a successful tool in areas where the endothelial cell-layer is affected by intervention from revascularization through angioplasty or stenting.



**Fig 13.** Bar graphs presenting results on contractility response from 2-MeSADP in murine aorta segments. Left bar graph shows no significant difference between control artery segments from vehicles vs. artery segments from mice pre-treated with clopidogrel per os. (P=n.s, n=5). Centre bar graph shows that ticagrelor (AZD6140) significantly blocking 2-MeSADP induced  $P2Y_{12}$  contraction on VSMC ( $59 \pm 7 \text{ vs. } 33 \pm 6 \ *P=0.015$ ). Right bar graph demonstrate results that marks ticagrelors ability to block  $P2Y_{12}$  contractions in murine arteries comparing to clopidogrel ( $64 \pm 8 \text{ vs. } 32 \pm 6 \ *P=0.002$ ).



**Fig 14**. Left Bar graph demonstrate results on human LIMA  $(29 \pm 7 \text{ vs. } 52 \pm 1, *P < 0.05, n=15)$  and small arteries  $(2 \pm 1 \text{ vs. } 12 \pm 3 **P=0.01, n=10)$  control vs. treated with ticagrelor (AZD6140), ex vivo. 2-MeSADP induced contractions are both reduced in LIMA and small human artery in the presence of ticagrelor.

# Succinate independently stimulates full platelet activation via cAMP and PI3β kinase signalling: (Paper III)

The results from a previous study, showing expression of SUCNR1 receptor in platelets, was confirmed using qPCR and the SUCNR1 gene was found to be transcribed at the same level as the one for P2Y<sub>1</sub>. The SUCNR1 expression on platelets was further confirmed on protein level by western blot and confocal imunofluorescence (Fig 15). Investigation with LTA confirmed that the effect of succinate mediated platelet activation was dependent on both TXA<sub>2</sub> generation and P2Y<sub>12</sub> activation as it was abolished in the presence of both ASA and ticagrelor. To evaluate if succinate activates any G $\alpha_{12/13}$ -protein we used a specific inhibitor to a downstream effector for the G $\alpha_{12/13}$ -protein, p160 Rho-associated coiled-coil containing kinase (Rho-kinase) p160ROCK [85]. Activation of the enzyme p160ROCK is important for the platelet shape change [85]. Pre-incubating platelets with the p160ROCK inhibitor Y27632 before succinate stimulation proved to have no effect on the platelet activation thus proving that succinate does not signal through G $\alpha_{12/13}$ -proteins.



Fig 15. Upper left bar graph: Expression of SUCNR1 on mRNA level studied through quantitative RT-PCR. The expression of SUCNR1,  $P2Y_{12}$  and A2a were normalized against  $P2Y_1$ . Lower left panel: Confocal immunofluorescense image platelets specific stained with rhodamine-6G (red) and SUCNR1 stained with primary goat polyclonal anti-SUCNR1 antibody (green). Right panel: Presence of

SUCNR1 on protein level in platelets (Plt) confirmed through western blot. Protein from Caki-1 cells (positive control) and Chinese Hamster Ovary (CHO) cells (negative control. GAPDH used as loading control.

We next evaluated if the platelet SUCNR1 is linked to the Gq-protein, which is a G-protein involved in the regulation of intracellular calcium. Our results showed that the intracellular calcium signaling from platelets was absent in the presence of succinate, and thus exclude the SUCNR1 as a Gaq-protein linked receptor. We then investigated if succinate is involved in the regulation of AC and hence the secondary messenger cAMP and its downstream effector VASP. We found that succinate suppresses generation of phosphorylated VASP as well as cAMP in platelets either stimulated by forskoline or prostaglandin  $E_1$  (PGE<sub>1</sub>). Furthermore, succinate stimulation increased both the levels of GPIIb/IIIa activation and the release of P-selectin (CD62P) in platelets (Fig 16).



**Fig 16**. Flow cytometry study on platelets. Left bar graph: GPIIb/IIIa activation in platelets. Marked differences between un-stimulated platelets and platelets stimulated with 3mM succinate or  $5\mu M$  ADP (ANOVA: \*\*P<0.01, n=16). Centre bar graph: P-Selectin release from platelets. Vesicle secretion of  $\alpha$ -granules mediated by succinate could be statistically confirmed between untreated platelets vs. platelets treated with 3mM succinate or  $5\mu M$  ADP (ANOVA: \*P<0.01, n=16). (C) Effects of succinate on forskoline induced cAMP-production in platelets. Significant differences in cAMP production was observed between platelets pretreated with 3mM succinate and 10 $\mu$ M forskolin vs. 10 $\mu$ M forskolin (ANOVA: \*\*P<0.001, n=4).

Besides inhibition of cAMP generation, Gi activation generates G<sub>βγ</sub> subunits that can regulate certain isoforms of PI3K. Blocking PI3K using a general PI3K inhibitor, wortmannin, resulted in a total block of succinate induced platelet stimulation, showing that SUCNR1 platelet activation is dependent on PI3-kinase

activation. Several isoforms of PI3K (PI3K $\alpha$ , PI3K $\beta$  and PI3K $\gamma$ ) are present in the human platelets [86]. We next evaluated which isoform of PI3K that was regulated by SUCNR1 activation. The results showed that platelets treated with a specific inhibitor for PI3K $\beta$  (TGX221) displayed significantly reduced aggregation upon stimulation with succinate. The PI3K initiates downstream phosporylation of the enzyme Akt1, also known as Protein Kinase B (PKB $\alpha$ ). Using western blot analysis we confirmed that succinate stimulated phosphorylation of PKB/Akt and that this effect could be primarily assigned to PI3K $\beta$  (Fig 17).



**Fig 17.** Western blot on washed platelets. Left fig: Presenting a representative result from a western blot on phosphorylated PKBa/Akt1 where the total content of PKBa/Akt1-protein serves as a loading control. Platelets treated with  $1\mu M$  of the  $PI3K\gamma$  inhibitor AS-604850, or  $1\mu M$  of the  $PI3K\beta$  inhibitor TGX221. All platelet samples, except the control, were stimulated with 3mM succinate. Right bar graph: Complete results from western blot where ratio is normalized towards control, (ANOVA: \*\*\*P < 0.001, \*\*P < 0.01, n = 3.

Our results also showed that the succinate stimulated SUCNR1 activated receptor responded in a biphasic manner and that the response was relative to the rising concentration of succinate with an EC<sub>50</sub> value equal to approximately 0.5 mM. This latter finding was confirmed by [<sup>35</sup>S]GTP $\gamma$ S-binding performed on isolated platelet membranes. [<sup>35</sup>S]GTP $\gamma$ S binding is, as previously described, a tool for functional receptor studies. The [<sup>35</sup>S]GTP $\gamma$ S-binding confirmed that SUCNR1 receptor activation in platelets is linked to the Gai protein. Furthermore, results from the [<sup>35</sup>S]GTP $\gamma$ S binding proved that the SUCNR1 receptor can be desensitized not only homologously by succinate, but also heterologously by ADP (Fig 18).



**Fig 18.** Dose-response of succinate-stimulated  $[{}^{35}S]GTP\gamma S$ -binding in platelet membranes.  $EC_{50}$ -value equals 110 $\mu$ M. untreated platelet membranes (**n**), platelet membranes pre-treated with 10 $\mu$ M 2MeSADP ( $\circ$ ), platelet membranes pre-treated with 3mM succinate (**•**). The maximal response, obtained for membranes pretreated with either 10 $\mu$ M 2MeSADP or 3mM succinate, was significantly reduced compared to untreated membranes (ANOVA: \*\*P<0.001, \*P<0.01, n=3).

More precise future functional studies on SUCNR1 activation demand better tools in the form of effective antagonists. SUCNR1 takes part in the platelet activation and might be an interesting target in the development of new antiplatelet therapies.

## Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y<sub>12</sub> receptor mediated platelet aggregation (Paper IV)

In this study we screened several of the intermediates of the mevalonate pathway for effects on platelet aggregation. One of them, farnesyl pyrophosphate (FPP) was found to have a blocking effect on ADP mediated platelet aggregation. Other platelet agonists like Thrombin Activating Peptide (TRAP) and epinephrine were also tested on FPP pre-incubated platelets, but without any difference in the inhibiting effect on platelet activation. However, FPPs inhibiting effect on ADP mediated platelet activation was further strengthened by the result from FACS on GPIIb/IIIa and P-selectin. Both GPIIb/IIIa and P-selectin activation was down-regulated compared to platelets stimulated only by ADP (Fig 17). Studies on cAMP generation also proved that FPP had a suppressing effect on ADP repressed forskoline stimulated cAMP production in platelets (Fig 19).



*Fig 19.* Bar graphs demonstrating ADP mediated platelet activation in presence of FPP. (A) GPIIb/IIIa activation, (B) P-selectin activation, (C) cAMP assay with FPP present.

To identify the mechanism of FPP effect on platelets, we studied  $[^{35}S]GTP\gamma S$  binding both on preparations of membranes from platelets and from  $P2Y_{12}$  transfected Chinese Hamster Ovary (CHO) -1 cells. The platelet membranes and the CHO-1-cells were preincubated with different concentrations of FPP before

stimulation with increasing concentrations of 2-MeSADP. The presence of FPP resulted in a decline in 2-MeSADP potency and also in a significant decrease in efficacy (Fig 20). These results prove that FPP interferes with the signaling of the  $P2Y_{12}$  receptor.



**Fig 20**.  $[{}^{35}S]GTP\gamma S$  binding assay on (**A**) platelet membranes and (**B**)  $P2Y_{12}$  transfected CHOK1-cells with different concentrations of FPP. (**■**) Control (2-MeSADP), (**□**) [FPP]=10µM, (**●**) [FPP]=50µM and (**○**) [FPP]=100µM

The most obvious similarity between FPP and ADP is the organization of both molecules into a pyrophosphate group and a hydrophobic backbone. However, the hydrophobic part of FPP consists of a 15-carbon chain that is quite different from the rings of ADP. In order to understand the way FPP and 2-MeSADP bind to the same receptor and to find the common rational basis for the binding modes, a molecular modeling study was conducted. (Fig 21). The results from the docking supported the hypothesis by showing that FPP is able to bind to the same binding cavity as ADP, sharing several aminoacid residues in the process.



**Fig 21.** Docking model of FPP and the  $P2Y_{12}$  receptor. (A) The 2-MeSADP molecule (green and with red phosphate group) docks with the receptor in the active site. (B) The FPP molecule (yellow and with red phosphate group) docks with the receptor in the active site. (C) Merge of 2-MeSADP and FPP.

The idea that FPP, besides its role in the mevalonate pathway, also have other functions is not new. FPP has also in an earlier report by Williams *et al.* [87] been identified as a potential ligand for the LPA<sub>5</sub> receptor, previously known as GPR92. It has also been shown, by Amisten *et al.* [33], that LPA<sub>5</sub>/GPR92 is present on mRNA level in platelets. It is likely that this receptor is responsible for the promoting effects of FPP on LPA mediated platelet shape-change. In this work we have identified FPP as an endogenous non-competitive antagonist on the P2Y<sub>12</sub> receptor. However, we have also noticed that low concentrations of FPP seem to have a potentiating effect on ADP mediated platelet activation. This suggests that FPP is a partial agonist which in the presence of ADP acts as an antagonist. The concentration of free FPP in plasma under normal and pathogenic conditions is still not known and could constitute the key to the role of FPP in the haemostatic/thrombotic pathway. At low concentrations FPP may act as a platelet activation.

## General Discussion

Mild hypothermia is used as complement method in the acute standard care treatment of patients affected by ACS or MI. In paper I we evaluated how mild hypothermia also influences the platelet activation. The study was performed in an *in vivo* milieu where maximal doses of the antiplatelet drug Plavix® (clopidogrel) were administrated, 600 mg *per os* in healthy volunteers. We found in this experiment that mild hypothermia does not attenuate platelet ADP-mediated response; on the contrary, we saw that mild hypothermia resulted in increased platelet activation.

The interaction between the vascular wall and platelets are central in the thrombotic process. The P2Y<sub>12</sub> receptor which is essential for platelet activation is also present on the vascular smooth muscle cell (VSMC) where it - if activated – can respond by initiating contractions. From this knowledge we initiated a comparative study (paper II) where we examined the possibility with a dual therapy. We compared the ability of two antiplatelet P2Y<sub>12</sub> receptor blocking substances (clopidogrel and ticagrelor) which both inhibit ADP induced platelet aggregation to inhibit ADP induced VSMC contractility in murine and human arteries. The results showed that the prodrug clopidogrel, the dominating P2Y<sub>12</sub>-blocking pharmaceutical treatment on the market, had no affect on P2Y<sub>12</sub> mediated contractility on VSMC *in vivo*. However ticagrelor, administrated *ex vivo*, proved to have the capability to decrease ADP mediated VSMC contractions. The platelets ability to activate is dependent on extracellular stimuli via membrane bound receptors. The extracellular signals are further amplified through several intracellular mechanisms.

In an earlier report by Amisten *et al.* [33] a number of membrane-bound receptors expressed at the platelet surface were identified, of which several were characterized as *orphan* GPCRs. In paper III we evaluated the intracellular response from one orphan GPCRs, the low affinity succinate receptor SUCNR1. SUCNR1 has in a previous report been identified as linked to both Gai and Gaq [46]. Succinate has in moderate concentrations proved to independently activate the platelet through SUCNR1-activation. However, the succinate activation requires TXA<sub>2</sub> generation and P2Y<sub>12</sub> activation for initiation of full scale platelet activation. Moreover, in a further examination of the intracellular signaling pathways of SUCNR1 it was able to couple to the Gai-protein and hence inhibit the membrane bound adenylate cyclase. The study also identified SUCNR1 as an activator for PI3 $\beta$ -kinase, an isoform of PI3-kinase. Earlier reports have confirmed that the isoform PI3K $\beta$  can both be regulated by G $\beta\gamma$ -subunits and by tyrosine kinases [88]. We believe that receptor mediated selection of PI3K isoforms is platelet specific and depend on the constellation of G $\beta$  and G $\gamma$ -subunits [89, 90].

FPP has earlier been reported by Williams *et al.* [87] to be an agonist on LPA<sub>5</sub> (GPR92) and thus have additive effects on LPA-mediated shape and change, a quality of FPP which agreed well with our findings. In an attempt to investigate if FPP or any other of the mevalonate intermediates had any effect on ADP mediated platelet aggregation, we screened the majority of them with LTA. Only FPP had any effect, and and was inhibitory. Also, LTA studies with epinephrine and TRAP confirmed that the inhibitory effect was true only for ADP induced platelet activation. Calcium-influx on washed platelets confirmed that ADP mediated P2Y<sub>1</sub> signaling was not attenuated in platelets pre-treated by 30µM FPP. However, the attenuated activation of P-selectin and PAC-1 in the presence of FPP together with the fact that FPP affected cAMP levels indicated that the purinergic receptor  $P2Y_{12}$  is the target for FPP. [<sup>35</sup>S]GTP $\gamma$ S binding assay performed on membranes isolated from human platelets as well as from P2Y<sub>12</sub>-transfected CHO-K1 cells showed that 2-MeSADP mediated P2Y<sub>12</sub> activation loses both potency and efficacy in presence of FPP. The molecular resemblance between the  $P2Y_{12}$ receptors endogenous agonist ADP and the FPP molecule is not obvious, but they both share a pyrophosphate group. A docking model between the P2Y<sub>12</sub> receptor and the synthetic stable ADP-analog 2-MeSADP and FPP concluded that FPP was able to fit into the active site of  $P2Y_{12}$  and thus attenuate the functionality of the receptor.

The studies throughout the thesis have aimed to investigate different aspects of platelet activation in haemostasis. The mechanisms that trigger the important and sensitive platelet activation are complex and sometimes they appear elusive. Studies on platelets are a vital tool for the understanding of how platelets fit into the haemostatic puzzle and hence, guide us to develop better antiplatelet therapies.

# Main Conclusions

- Mild hypothermia does not attenuate platelet activation. Hypothermia has an augmenting effect on ADP induced platelet activation.
- Hypothermia induced clopidogrel resistance is reduced in the presence of the P2Y<sub>12</sub> receptor blocking substance ticagrelor (AZD6140).
- Mild hypothermia can not independently be used clinically as an anti-platelet therapy. Instead patients treated with mild hypothermia might even need more potent antagonists.
- Clopidogrel administrated *in vivo* have no inhibiting effect on ADP induced VSMC contractions in murine aorta and human LIMA or small arteries. However, ticagrelor, *ex vivo* has the ability to block ADP mediated VSMC contractions.
- Succinate has *per se* the capacity to induce full platelet activation. Succinate activation is dependent of TXA<sub>2</sub> generation, ADP mediated activation of the P2Y<sub>12</sub> receptor, and PI3Kβ activation.
- The intracellular signaling pathways of the succinate receptor, SUCNR1, inhibit adenylate cyclase cAMP production and regulate Akt1 activation through phosphorylation by the PI3K isoform PI3Kβ.
- FPP is an endogenous antagonist of the  $P2Y_{12}$  receptor in high concentrations (>30 $\mu$ M), but seems to have a potentiating effect in ADP mediated platelet activation if present at low concentrations (partial agonist and antagonist).

## Populärvetenskaplig sammanfattning

Hjärt- och kärlsjukdomar är den främsta orsaken till en för tidig död för både män och kvinnor i dagens industrialiserade samhällen. I det akuta förloppet av dessa sjukdomar spelar blodplätten (trombocyten) en viktig roll då den är inblandad i bildandet av blodproppar. Den kärnlösa och kortlivade trombocytens roll är vanligen en annan och central i läkningsprosessen av skadade blodkärl. Vid en kärlskada organiserar sig trombocyterna med trådverk av samverkande proteiner för att levra (koagulera) blodet och därmed blodförlust. Trombocyterna använder sig av ytbundna receptorer och frisättning av signalmolekyler för att kommunisera med sin omgivning. En av dessa receptorer, P2Y<sub>12</sub> är viktig för att aktivera trombocyten. P2Y<sub>12</sub> finns även på ytan av glatt muskulatur i blodkärlsväggen, där den svarar med att kontrahera muskelcellen. Flera läkemedel, däribland clopidogrel och ticagrelor har utvecklats för att förhindra att P2Y<sub>12</sub> aktiveras. Clopidogrel måste omvandlas via levern för att aktiveras, vilket innebär att clopidogrel föreligger i låga koncentrationer i blodplasman. Clopidogrel har också påvisats ha en låg effektivitet vid behandling. Ticagrelor är en substans som tillhör den nya generationen P2Y<sub>12</sub> blockerare som visat sig ha en bättre blockerande effekt på trombocyter än sina föregångare.

Det finns ett behov av att ta fram nya terapier för att sänka trombocytaktiveringen. Genom att sänka kroppstemperaturen från 37C till 33C (mild hypotermi) sänks kroppsmetabolismen och behovet av syre till organ och vävnader. Detta har gett positiva resultat vid hjärt- och kärlkirurgi, och visat sig vara en metod för att skona vävnad strax innan samt under de kirurgiska ingreppen. Dock har studier på trombocytens svar vid mild hypotermi visat på både en ökad respektive minskad aktivering. Vid en undersökning kunde vi konstatera en ökad trombocyt aktivering vid 33C jämfört med 37C. Vid samma undersökning kunde vi bekräfta att även effektiviteten hos clopidogrel sänktes vid 33C jämfört med 37C.

Eftersom  $P2Y_{12}$  receptorn även uttrycks i glatt muskulatur så skulle i teorin en blockerande substans för  $P2Y_{12}$  receptorn, antingen clopidogrel eller ticagrelor kunna blockera dess aktivering på muskelcellerna i kärlen och förhindra skadliga kärlsammandragningar. Våra undersökningar visade att kontraktionerna i den glatta muskulaturen hämmades utav ticagrelor, medan clopidogrel var verkningslöst. Vi har vid tidigare undersökningar på trombocyter identifierat höga transkriptnivåer för succinatreceptorn (SUCNR1). Vid vidare undersökningar upptäckte vi att denna receptor fanns uttryckt på trombocytens yta och har en egen såväl som förstärkande effekt på trombocyt-aktivering. Aktivering av SUCNR1 visade sig vara beroende av  $P2Y_{12}$  aktivering, frisättning av tromboxan  $A_2$ , såväl som det SUCNR1 aktiverade PI3K $\beta$  enzymet.

Ett något oväntat fynd gjordes vid en undersökning av signalmolekyler, inblandade i kolesterol-syntesen. Det visade sig att en av signalmolekylerna, farnesyl pyrofosfat (FPP), uppvisade en hämmande effekt på ADP medierad trombocyt aktivering. Preliminära försök visade att det endast var den ADP aktiverade  $P2Y_{12}$ -receptorn som blockerades genom närvaro av FPP. Vid senare underökningar kunde vi bekräfta att FPP, vid moderata koncentrationer, verkade hämmande på  $P2Y_{12}$ -receptorn, men också att FPP vid låga koncentrationer kan fungera additivt på ADP/P2Y\_12-medierad trombocyt-aktivering.

Sammanfattningsvis har dessa arbeten, var och en på sitt vis, verkat för en kliniskt och prekliniskt ökad kunskap om trombocytaktivering. Genom studierna angående trombocytaktivering under påverkan av mild hypotermi, har vi visat att kyla ökar trombocytaktivering samt att clopidogrel förlorar en del av sin P2Y<sub>12</sub>-blockerande effekt. Den kliniska relevansen består i att behålla doserna av anti-trombotiska läkemedel även i de fall där mild kylning används som ett komplement vid operativa ingrepp. Vidare har våra försök visat att P2Y<sub>12</sub>-hämmaren ticagrelor även har en hämmande effekt på ADP stimulerad P2Y<sub>12</sub>-receptor inducerad kärlkontraktion, vilket kan vara en viktig terapeutisk egenskap i de fall där kärlväggen är skadad med frilagd underliggande glatt muskulatur. Vi har också identifierat succinat receptorns bakomliggande funktioner för medverkan i trombocytaktivering. Till sist har våra studier visat en ny roll hos en viktig molekyl i kolesterol syntesen, farnesyl pyrofosfat, nämligen den att farnesyl pyrofosfat även visat på antagonistiska- såväl som agonistiska egenskaper på den för trombocyt-aktivering centrala P2Y<sub>12</sub> receptorn.

## Acknowledgements

I would like to express my sincerely gratitude to all persons who have supported me and helped me through the years as a PhD student. This thesis would not been accomplished without all those lending arms. I especially which to address warm thanks to:

My supervisor Professor **David Erlinge** for introducing me into the world of purinergic receptor signaling. You are truly an encouraging and inspiring person who rather se possibilities than problems. Thanks for all your support and patience in every aspect of my time in your group; it has truly been a roller-coaster experience over the years.

My co-supervisor **Björn Olde** for all your theoretical and practical support. You are truly a brilliant scientist and especially in the field of pharmacology. Thanks for all those interesting and funny conversations that we've had during the years.

Our lab technician and my room-mate, **Siv Svensson** for all your help, endurance and needle-expertise in the context of blood collection. Without all these blooddraining moments, this and I guess several coming PhD thesis would not be possible. Furthermore, thank you for all those nice conversations we have had trough the years.

**Chanyuan Tan** for your patience and accuracy with western blots in our laboratory collaboration. I wish you good luck in your forthcoming PhD work.

**Olof Gidlöf** for your skillful experience with FACS and RT-PCR in our laboratory collaboration. Thanks for good conversations about bread-baking in general and sourdough in particular. I whish you good luck in your further PhD-work.

**Jenny Nilsson-Öhlund** for all your expertise and helpful assistance with confocal imaging and western blots though I know that you have your hands full. You are an asset to our group.

**Oscar Braun** for your inspiering ideas and fruitfull collaboration about platelets, where you tought me how to use LTA and FACS.

Our research administrator **Monica Magnusson** for all your helpful assistance with small as well as big problems during the years – always with a cheerful smile.

**Francesca Deflorian** and **Kenneth A Jacobson** at the National Institute of Diabetes and Digestive and Kidney Diseases, NIH, U.S.A. for cooperation and for your great FPP docking studies on P2Y<sub>12</sub>.

**Persons on the department of cardiology and BMC C12 and D12** for help and support in every sense and being freely voluntarily persons in providing me with raw-material for my bloody experiments. Thanks all of you for inspiring and fun "Fika"-discussions, making my PhD time amusing. No one mentioned, no one forgotten.

My parents, my sister with family and aunt Birgitta for being there for me.

**Karins family and relatives** for your never ending support and belief in me during all these years. What can I say, words can't describe my appreciation. You are fantastic and very dear to me.

Finally, I would like to direct a very special warm and loving thanks to my wonderful wife **Karin**. I could not ask for a better companion in life. You make it happen. **Oscar, Alexander** and **Lykke** I love you so much and you constantly remind me about the true values of life.

## References

- 1. Hansson, G.K., A.K. Robertson, and C. Soderberg-Naucler, *Inflammation and atherosclerosis*. Annu Rev Pathol, 2006. **1**: p. 297-329.
- 2. Anversa, P. and E.H. Sonnenblick, *Ischemic cardiomyopathy:* pathophysiologic mechanisms. Prog Cardiovasc Dis, 1990. **33**(1): p. 49-70.
- 3. Erlinge, D. and G. Burnstock, *P2 receptors in cardiovascular regulation and disease*. Purinergic Signal, 2008. **4**(1): p. 1-20.
- 4. Libby, P., *Changing concepts of atherogenesis*. J Intern Med, 2000. **247**(3): p. 349-58.
- 5. Volpe, M., D. Chin, and F. Paneni, *The challenge of polypharmacy in cardiovascular medicine*. Fundam Clin Pharmacol, 2010. **24**(1): p. 9-17.
- 6. Sowden, G.L. and J.C. Huffman, *The impact of mental illness on cardiac outcomes: a review for the cardiologist.* Int J Cardiol, 2009. **132**(1): p. 30-7.
- 7. Krishna, S.M., et al., *Genetic and epigenetic mechanisms and their possible role in abdominal aortic aneurysm.* Atherosclerosis, 2010. **212**(1): p. 16-29.
- 8. Kruth, H.S., *The fate of lipoprotein cholesterol entering the arterial wall.* Curr Opin Lipidol, 1997. **8**(5): p. 246-52.
- Hajjar, D.P. and M.E. Haberland, Lipoprotein trafficking in vascular cells. Molecular Trojan horses and cellular saboteurs. J Biol Chem, 1997. 272(37): p. 22975-8.
- 10. Faggiotto, A. and R. Ross, *Studies of hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque.* Arteriosclerosis, 1984. **4**(4): p. 341-56.
- 11. Schonbeck, U. and P. Libby, *CD40 signaling and plaque instability*. Circ Res, 2001. **89**(12): p. 1092-103.
- 12. Varga, J., J. Rosenbloom, and S.A. Jimenez, *Transforming growth factor* beta (TGF beta) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. Biochem J, 1987. **247**(3): p. 597-604.
- 13. Di Lullo, G.A., et al., *Mapping the ligand-binding sites and diseaseassociated mutations on the most abundant protein in the human, type I collagen.* J Biol Chem, 2002. **277**(6): p. 4223-31.
- 14. Libby, P., *Molecular bases of the acute coronary syndromes*. Circulation, 1995. **91**(11): p. 2844-50.

- 15. Hollopeter, G., et al., *Identification of the platelet ADP receptor targeted by antithrombotic drugs*. Nature, 2001. **409**(6817): p. 202-7.
- 16. Gharacholou, S.M., et al., *Antithrombotic therapy in acute coronary syndromes: guidelines translated for the clinician.* J Thromb Thrombolysis, 2010. **29**(4): p. 516-28.
- 17. *Guidelines on myocardial revascularization*. Eur J Cardiothorac Surg, 2010. **38 Suppl**: p. S1-S52.
- 18. Hudson, P.A., M.S. Kim, and J.D. Carroll, *Coronary ischemia and percutaneous intervention*. Cardiovasc Pathol, 2010. **19**(1): p. 12-21.
- 19. De Luca, G. and P. Marino, Antithrombotic therapies in primary angioplasty: rationale, results and future directions. Drugs, 2008. **68**(16): p. 2325-44.
- 20. Anderson, J.L., et al., ACC/AHA 2007 guidelines for the management of patients with unstable angina/non-ST-Elevation myocardial infarction: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to *Revise the 2002 Guidelines for the Management of Patients With Unstable* Angina/Non-ST-Elevation Mvocardial Infarction) developed in collaboration with the American College of Emergency Physicians, the Society for Cardiovascular Angiography and Interventions, and the Society of Thoracic Surgeons endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation and the Society for Academic Emergency Medicine. J Am Coll Cardiol, 2007. 50(7): p. e1e157.
- 21. Waters, D.D. and I. Ku, *Early statin therapy in acute coronary syndromes: the successful cycle of evidence, guidelines, and implementation.* J Am Coll Cardiol, 2009. **54**(15): p. 1434-7.
- 22. Hartwig, J.H., et al., *The elegant platelet: signals controlling actin assembly*. Thromb Haemost, 1999. **82**(2): p. 392-8.
- 23. Italiano, J.E., Jr. and R.A. Shivdasani, *Megakaryocytes and beyond: the birth of platelets.* J Thromb Haemost, 2003. **1**(6): p. 1174-82.
- 24. Youssefian, T. and E.M. Cramer, *Megakaryocyte dense granule components are sorted in multivesicular bodies*. Blood, 2000. **95**(12): p. 4004-7.
- 25. Watson, S.P., *Platelet activation by extracellular matrix proteins in haemostasis and thrombosis.* Curr Pharm Des, 2009. **15**(12): p. 1358-72.
- 26. Roberts, D.E., A. McNicol, and R. Bose, *Mechanism of collagen activation in human platelets*. J Biol Chem, 2004. **279**(19): p. 19421-30.
- 27. Stenberg, P.E., et al., *Prolonged bleeding time with defective platelet filopodia formation in the Wistar Furth rat.* Blood, 1998. **91**(5): p. 1599-608.
- 28. Mitchell, J.A., et al., *Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium.* Exp Physiol, 2008. **93**(1): p. 141-7.

- 29. Rijken, D.C. and H.R. Lijnen, New insights into the molecular mechanisms of the fibrinolytic system. J Thromb Haemost, 2009. 7(1): p. 4-13.
- 30. Oury, C., et al., *The platelet ATP and ADP receptors*. Curr Pharm Des, 2006. **12**(7): p. 859-75.
- 31. Ebbeling, L., et al., *Rapid ultrastructural changes in the dense tubular system following platelet activation*. Blood, 1992. **80**(3): p. 718-23.
- 32. Jardin, I., et al., Intracellular calcium release from human platelets: different messengers for multiple stores. Trends Cardiovasc Med, 2008. 18(2): p. 57-61.
- 33. Amisten, S., et al., *Gene expression profiling for the identification of Gprotein coupled receptors in human platelets.* Thromb Res, 2008. **122**(1): p. 47-57.
- 34. Chen, J., et al., *Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice*. Blood, 2004. **104**(6): p. 1703-10.
- 35. Baurand, A., et al., *Inhibition of platelet function by administration of MRS2179, a P2Y1 receptor antagonist*. Eur J Pharmacol, 2001. **412**(3): p. 213-21.
- 36. Cattaneo, M., et al., *Antiaggregatory activity in human platelets of potent antagonists of the P2Y 1 receptor*. Biochem Pharmacol, 2004. **68**(10): p. 1995-2002.
- 37. Popma, J.J., et al., *Antithrombotic therapy during percutaneous coronary intervention: the Seventh ACCP Conference on Antithrombotic and Thrombolytic Therapy*. Chest, 2004. **126**(3 Suppl): p. 576S-599S.
- 38. Leonardi, S., et al., *Rationale and design of the randomized, double-blind trial testing INtraveNous and Oral administration of elinogrel, a selective and reversible P2Y(12)-receptor inhibitor, versus clopidogrel to eVAluate Tolerability and Efficacy in nonurgent Percutaneous Coronary Interventions patients (INNOVATE-PCI).* Am Heart J, 2010. **160**(1): p. 65-72.
- 39. van Giezen, J.J., et al., Comparison of ticagrelor and thienopyridine P2Y(12) binding characteristics and antithrombotic and bleeding effects in rat and dog models of thrombosis/hemostasis. Thromb Res, 2009. **124**(5): p. 565-71.
- 40. Jaremo, P., et al., *Individual variations of platelet inhibition after loading doses of clopidogrel.* J Intern Med, 2002. **252**(3): p. 233-8.
- 41. Cattaneo, M., *Aspirin and clopidogrel: efficacy, safety, and the issue of drug resistance*. Arterioscler Thromb Vasc Biol, 2004. **24**(11): p. 1980-7.
- 42. Hogberg, C., et al., *The reversible oral P2Y12 antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature.* Int J Cardiol, 2010. **142**(2): p. 187-92.
- 43. Cannon, C.P., et al., *Comparison of ticagrelor with clopidogrel in patients with a planned invasive strategy for acute coronary syndromes (PLATO): a randomised double-blind study.* Lancet, 2010. **375**(9711): p. 283-93.

- 44. Cannon, C.P., et al., Safety, tolerability, and initial efficacy of AZD6140, the first reversible oral adenosine diphosphate receptor antagonist, compared with clopidogrel, in patients with non-ST-segment elevation acute coronary syndrome: primary results of the DISPERSE-2 trial. J Am Coll Cardiol, 2007. **50**(19): p. 1844-51.
- 45. Wittenberger, T., H.C. Schaller, and S. Hellebrand, *An expressed sequence* tag (*EST*) data mining strategy succeeding in the discovery of new *G*-protein coupled receptors. J Mol Biol, 2001. **307**(3): p. 799-813.
- 46. He, W., et al., *Citric acid cycle intermediates as ligands for orphan G*protein-coupled receptors. Nature, 2004. **429**(6988): p. 188-93.
- 47. Krebs, H.A., *The history of the tricarboxylic acid cycle*. Perspect Biol Med, 1970. **14**(1): p. 154-70.
- 48. Hakak, Y., et al., *The role of the GPR91 ligand succinate in hematopoiesis.* J Leukoc Biol, 2009. **85**(5): p. 837-43.
- 49. Toma, I., et al., *Succinate receptor GPR91 provides a direct link between high glucose levels and renin release in murine and rabbit kidney.* J Clin Invest, 2008. **118**(7): p. 2526-34.
- 50. Sadagopan, N., et al., *Circulating succinate is elevated in rodent models of hypertension and metabolic disease*. Am J Hypertens, 2007. **20**(11): p. 1209-15.
- 51. Correa, P.R., et al., *Succinate is a paracrine signal for liver damage*. J Hepatol, 2007. **47**(2): p. 262-9.
- 52. Wust, J., Presumptive diagnosis of anaerobic bacteremia by gas-liquid chromatography of blood cultures. J Clin Microbiol, 1977. **6**(6): p. 586-90.
- 53. Huang, E.M., E.B. McGowan, and T.C. Detwiler, *Succinate potentiates the action of platelet agonists*. Thromb Res, 1984. **36**(1): p. 1-8.
- 54. Goldstein, J.L. and M.S. Brown, *Regulation of the mevalonate pathway*. Nature, 1990. **343**(6257): p. 425-30.
- 55. Gibbs, J.B. and A. Oliff, *The potential of farnesyltransferase inhibitors as cancer chemotherapeutics*. Annu Rev Pharmacol Toxicol, 1997. **37**: p. 143-66.
- 56. Agrawal, A.G. and R.R. Somani, *Farnesyltransferase inhibitor as anticancer agent.* Mini Rev Med Chem, 2009. **9**(6): p. 638-52.
- 57. Lancet, J.E., et al., *A phase 2 study of the farnesyltransferase inhibitor tipifarnib in poor-risk and elderly patients with previously untreated acute myelogenous leukemia.* Blood, 2007. **109**(4): p. 1387-94.
- 58. Oh, D.Y., et al., *Identification of farnesyl pyrophosphate and N-arachidonylglycine as endogenous ligands for GPR92*. J Biol Chem, 2008. **283**(30): p. 21054-64.
- 59. Bang, S., et al., *Farnesyl pyrophosphate is a novel pain-producing molecule via specific activation of TRPV3*. J Biol Chem, 2010. **285**(25): p. 19362-71.
- 60. Braunwald, E., et al., ACC/AHA guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial

infarction--2002: summary article: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on the Management of Patients With Unstable Angina). Circulation, 2002. **106**(14): p. 1893-900.

- 61. Bernard, S.A., et al., *Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia.* N Engl J Med, 2002. **346**(8): p. 557-63.
- 62. Bernard, S.A., *Therapeutic hypothermia after cardiac arrest*. Med J Aust, 2004. **181**(9): p. 468-9.
- 63. Holzer, M., et al., *Hypothermia for neuroprotection after cardiac arrest: systematic review and individual patient data meta-analysis.* Crit Care Med, 2005. **33**(2): p. 414-8.
- 64. Lagneau, F., et al., *Effect of mild hypothermia on the vascular actions of phenylephrine in rat aortic rings.* Br J Anaesth, 1999. **82**(6): p. 938-40.
- 65. Michelson, A.D., et al., *Reversible inhibition of human platelet activation* by hypothermia in vivo and in vitro. Thromb Haemost, 1994. **71**(5): p. 633-40.
- 66. Frelinger, A.L., 3rd, et al., *Combined effects of mild hypothermia and glycoprotein IIb/IIIa antagonists on platelet-platelet and leukocyte-platelet aggregation.* Am J Cardiol, 2003. **92**(9): p. 1099-101.
- 67. Lindenblatt, N., et al., *Sustained hypothermia accelerates microvascular thrombus formation in mice*. Am J Physiol Heart Circ Physiol, 2005. **289**(6): p. H2680-7.
- 68. Faraday, N. and B.A. Rosenfeld, *In vitro hypothermia enhances platelet GPIIb-IIIa activation and P-selectin expression*. Anesthesiology, 1998. **88**(6): p. 1579-85.
- 69. Xavier, R.G., et al., *Enhanced platelet aggregation and activation under conditions of hypothermia.* Thromb Haemost, 2007. **98**(6): p. 1266-75.
- 70. Elwood, P.C., et al., *Temperature and risk factors for ischaemic heart disease in the Caerphilly prospective study.* Br Heart J, 1993. **70**(6): p. 520-3.
- 71. Gachet, C., *Regulation of platelet functions by P2 receptors*. Annu Rev Pharmacol Toxicol, 2006. **46**: p. 277-300.
- 72. Born, G.V., *Aggregation of blood platelets by adenosine diphosphate and its reversal.* Nature, 1962. **194**: p. 927-9.
- 73. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.
- 74. Frojmovic, M., T. Wong, and T. van de Ven, *Dynamic measurements of the platelet membrane glycoprotein IIb-IIIa receptor for fibrinogen by flow cytometry. I. Methodology, theory and results for two distinct activators.* Biophys J, 1991. **59**(4): p. 815-27.
- 75. Horstrup, K., et al., *Phosphorylation of focal adhesion vasodilator*stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. Eur J Biochem, 1994. **225**(1): p. 21-7.

- 76. Galler, A.B., et al., *VASP-dependent regulation of actin cytoskeleton rigidity, cell adhesion, and detachment.* Histochem Cell Biol, 2006. **125**(5): p. 457-74.
- 77. Blann, A.D., S.K. Nadar, and G.Y. Lip, *The adhesion molecule P-selectin and cardiovascular disease*. Eur Heart J, 2003. **24**(24): p. 2166-79.
- 78. Varga-Szabo, D., A. Braun, and B. Nieswandt, *Calcium signaling in platelets*. J Thromb Haemost, 2009. **7**(7): p. 1057-66.
- 79. Sato, M., et al., *Accessory proteins for G proteins: partners in signaling*. Annu Rev Pharmacol Toxicol, 2006. **46**: p. 151-87.
- 80. Harrison, C. and J.R. Traynor, *The [35S]GTPgammaS binding assay:* approaches and applications in pharmacology. Life Sci, 2003. **74**(4): p. 489-508.
- 81. Malmsjo, M., L. Edvinsson, and D. Erlinge, *P2U-receptor mediated* endothelium-dependent but nitric oxide-independent vascular relaxation. Br J Pharmacol, 1998. **123**(4): p. 719-29.
- 82. Wihlborg, A.K., et al., *Extracellular nucleotides induce vasodilatation in human arteries via prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor.* Br J Pharmacol, 2003. **138**(8): p. 1451-8.
- 83. Kasakov, L. and G. Burnstock, *The use of the slowly degradable analog, alpha, beta-methylene ATP, to produce desensitisation of the P2purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder.* Eur J Pharmacol, 1982. **86**(2): p. 291-4.
- 84. Wihlborg, A.K., et al., *ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels.* Arterioscler Thromb Vasc Biol, 2004. **24**(10): p. 1810-5.
- 85. Paul, B.Z., et al., *Dynamic regulation of microtubule coils in ADP-induced platelet shape change by p160ROCK (Rho-kinase).* Platelets, 2003. **14**(3): p. 159-69.
- 86. Hirsch, E., et al., *Resistance to thromboembolism in PI3Kgamma-deficient mice*. FASEB J, 2001. **15**(11): p. 2019-21.
- 87. Williams, J.R., et al., Unique ligand selectivity of the GPR92/LPA5 lysophosphatidate receptor indicates role in human platelet activation. J Biol Chem, 2009. **284**(25): p. 17304-19.
- 88. Maier, U., A. Babich, and B. Nurnberg, *Roles of non-catalytic subunits in gbetagamma-induced activation of class I phosphoinositide 3-kinase isoforms beta and gamma.* J Biol Chem, 1999. **274**(41): p. 29311-7.
- 89. Dupre, D.J., et al., *The role of Gbetagamma subunits in the organization, assembly, and function of GPCR signaling complexes.* Annu Rev Pharmacol Toxicol, 2009. **49**: p. 31-56.
- 90. Ford, C.E., et al., *Molecular basis for interactions of G protein betagamma subunits with effectors.* Science, 1998. **280**(5367): p. 1271-4.

# Appendix: Original Papers I-IV

- I. Carl Högberg, David Erlinge and Oscar Ö Braun Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment *Thrombosis Journal 2009; 7:2*
- II. Carl Högberg, Helen Svensson, Ronny Gustafsson; Atli Eyjolfsson, and David Erlinge
   The reversible oral P2Y(12) antagonist AZD6140 inhibits
   ADP-induced contractions in murine and human vasculature

  International Journal of Cardiology 2010, vol 142 pp 187-192
- III. Carl Högberg, Olof Gidlöf, Chanyuan Tan, Siv Svensson, Jenny Nilsson-Öhman, David Erlinge and Björn Olde Succinate independently stimulates full platelet activation via cAMP and PI3β kinase signaling Journal of Thrombosis and Haemostasis (in press)
- IV. Carl Högberg, Olof Gidlöf, Francesca Deflorian, Kenneth A. Jacobson, Björn Olde and David Erlinge Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y<sub>12</sub> receptor mediated platelet aggregation (*Manuscript*)

# Paper I

## **Thrombosis Journal**

Original basic research

Bio Med Central

#### **Open Access**

### Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment

Carl Högberg, David Erlinge\* and Oscar Ö Braun

Address: Department of Cardiology, Lund University, Lund, Sweden

Email: Carl Högberg - carl.hogberg@med.lu.se; David Erlinge\* - david.erlinge@med.lu.se; Oscar Ö Braun - oscar.braun@med.lu.se \* Corresponding author

Published: 23 February 2009

Thrombosis Journal 2009, 7:2 doi:10.1186/1477-9560-7-2

This article is available from: http://www.thrombosisjournal.com/content/7/1/2

© 2009 Högberg et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<u>http://creativecommons.org/licenses/by/2.0</u>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Received: 19 November 2008 Accepted: 23 February 2009

#### Abstract

**Background:** Mild hypothermia is currently standard of care for cardiac arrest patients in many hospitals and a common belief is that hypothermia attenuates platelet aggregation. We wanted to examine the effects of clopidogrel on platelet aggregation during hypothermia.

**Methods:** Platelet reactivity at 37°C and 33°C was evaluated by light transmission aggregometry and vasodilator-stimulated phosphoprotein (VASP) in blood from healthy volunteers before, and 24 hours after, a 600 mg loading dose of clopidogrel.

**Results:** Collagen, 5-HT, epinephrine, U46619 and ADP-induced platelet aggregation was unaltered or even increased by hypothermia. After clopidogrel, there was a significant increase in platelet aggregation for 5 and 20  $\mu$ M ADP at 33°C compared to 37°C (46 ± 5 vs. 34 ± 5% and 58 ± 4 vs. 47 ± 4%, p < 0.001, n = 8). Hypothermia also increased ADP-induced aggregation after pretreatment with the P2Y<sub>1</sub> antagonist MRS2500. The decreased responsiveness to clopidogrel during hypothermia could be overcome by addition of the reversible P2Y<sub>12</sub> antagonist AZD6140. ADP-induced inhibition of VASP-phosphorylation was unaffected by hypothermia both in the presence and absence of clopidogrel. A dose-response curve for ADP-induced platelet aggregation revealed increased potency for ADP during hypothermia with no difference in efficacy.

**Conclusion:** Mild hypothermia did not attenuate platelet aggregation, instead it even increased ADP-stimulated platelet aggregation after clopidogrel treatment. Dual platelet inhibition with aspirin and a  $P2Y_{12}$  receptor antagonist is probably needed for patients with acute coronary syndromes treated with mild hypothermia, and it is possible that future ADP blockers could be of benefit.

#### Introduction

Hypothermia is a condition in which many biological reactions are altered. Even a minor change of temperature in a cell can alter the response to stimuli. Mild hypothermia (33–35°C) has been shown to reduce mortality and improve neurological outcome in unconscious patients

with cardiac arrest [1,2], and is recommended by treatment guidelines [3]. Mild hypothermia is already standard of care in many hospitals for cardiac arrest patients. Furthermore, mild hypothermia has been shown to reduce myocardial infarct size in animal models, and clinical studies are ongoing to determine whether this strategy can preserve not only the brain but also the heart [4]. For many patients with cardiac arrest, the aetiology is an acute coronary syndrome, which needs to be treated with platelet inhibitors. The cornerstone of this treatment is dual antiplatelet therapy with aspirin and clopidogrel [3]. However, to our knowledge, the pharmacodynamic effects of clopidogrel during hypothermia have never been studied.

Conventional wisdom holds that hypothermia reduces platelet activation, and it has been suggested that antiplatelet treatment should be reduced during hypothermia. There are some studies supporting this notion [5-7]. However, there are more recent reports suggesting increased platelet reactivity during mild hypothermia [8-11]. It is of great importance to select the correct level of platelet inhibition for this patient category. Insufficient platelet inhibition, and cardiac death. It is therefore important to understand the effect of hypothermia on clopidogrel treatment.

To examine the effect of clopidogrel during mild hypothermia, we analyzed platelet reactivity as measured by light transmission aggregometry (LTA), vasodilator-stimulated phosphoprotein (VASP)-phosphorylation, and a  $P2Y_{12}$  specific flow cytometry assay, in healthy volunteers at 37° and 33°C before and 24 hours after a 600 mg dose of clopidogrel.

#### Materials and methods Platelet preparation for LTA analysis

Whole blood samples (40 mL) were collected from healthy voluntary blood donors (n = 8). The blood samples were collected from an antecubital vein into Becton Dickinson (BD) Vacutainer<sup>™</sup> tubes containing 0.129 M sodium citrate. A second blood sample was collected 24 hours after a 600 mg oral loading dose of clopidogrel. To obtain platelet rich plasma (PRP), whole blood was centrifuged (10 min, 140 × g, room temperature (RT)). After centrifugation the supernatant containing the PRP was collected and transferred to a 15 mL polypropylene tube. To obtain reference platelet poor plasma (PPP), whole blood was centrifuged (10 min, 2260 × g, RT). The supernatant containing PPP was collected into a 15 mL polypropylene tube.

#### LTA analyses

Two serial connected aggregometers (Chrono-Log 490, Chrono-Log Corporation, Havertown, PA, USA) were preheated to 37°C and 33°C respectively. To allow for temperature equilibration, the PRP was incubated for 5 minutes in the aggregometers prior to addition of an agonist. In experiments with ex vivo antagonists, addition of these compounds was followed by incubation for 5 min prior to ADP stimulation. Analyses were performed using AGGRO/LINK<sup>®</sup> software (Chrono-Log Corporation, Havertown, PA, USA.). Stirring was set to 1200 rpm and the optical band was in the range of 600 OD. Each of the experiments continued for 6 minutes (to observe if a secondary aggregation took place). The test volumes containing PRP were set to 250 µL, while the PPP volume in the reference wells of the two aggregometers was set to 500 µL.

#### Flow cytometric analysis of VASP-phosphorylation

Whole blood was collected from the antecubital vein into BD Vacutainer<sup>™</sup> tubes containing 0.129 M sodium citrate. The blood was incubated for 10 min at 33°C and 37°C. The VASP assay was performed using the platelet VASP/ P2Y<sub>12</sub> kit (Biocytex Platelet VASP kit, Marseille, FR). The experimental procedure was carried out according to the manufacturer's instructions except that the samples were prepared at 33°C and 37°C instead of room temperature until the fixation step. Mean Fluorescence Intensity (MFI) was measured with a flow cytometer (FACScalibur<sup>\*</sup>, BD, USA). Platelet reactivity index (PRI) was calculated from the corrected MFI (cMFI) of prostaglandin E<sub>1</sub> (PGE<sub>1</sub>) and ADP- and PGE<sub>1</sub>- treated samples according to the following equation:

 $PRI = [(cMFI_{(PGE1)} - cMFI_{(PGE1+ADP)})/cMFI_{(PGE1)}] \times 100$ 

#### Drugs

ADP, epinephrine, collagen, and thrombin were from Chrono-Log Corporation, USA. AZD6140 was a gift from Astra-Zeneca, Sweden. MRS2500 was from Tocris Bioscience, UK. U-46619 was from Sigma-Aldrich, USA. Clopidogrel was from Sanofi Pharma Bristol-Myers Squibb SNC, France. All drugs (clopidogrel excluded) were dissolved in 0.9% saline; AZD6140 was dissolved in DMSO at 10<sup>-</sup>2 M and then further diluted in 0.9% saline.

#### Ethics

The Ethics Committee of Lund University approved the project. All blood donors provided signed informed consent to participate in the study.

#### Calculation and statistics

Statistical analyses were performed using the GraphPad Prism 4.0 software (Graph Pad Software, USA). LTA and VASP data were analyzed using paired Student's t-test. Pvalues less than 0.05 were regarded as statistically significant. Values are presented as mean  $\pm$  s.e.m.

#### Results

#### ADP-stimulated platelet aggregation evaluated by LTA

There was no significant difference in maximum platelet aggregation (MPA) levels when PRP was stimulated with  $5 \mu$ M ADP at  $33 \degree$ C compared to  $37 \degree$ C ( $79 \pm 1 \text{ vs. } 80 \pm 1\%$ ,

P = NS, n = 8) or with 20  $\mu$ M ADP at 33 °C compared to 37 °C (80  $\pm$  2 vs. 80  $\pm$  1%, P = NS, n = 8) [Figure 1a, b].

### ADP-stimulated platelet aggregation before and after clopidogrel treatment

24 hours after a loading dose of 600 mg clopidogrel, there was a significant increase in MPA after both 5 and 20  $\mu$ M ADP at 33 °C compared to 37 °C (46 ± 5 vs. 34 ± 5%, P < 0.001, n = 8, and 58 ± 4 vs. 47 ± 4%, P < 0.001, n = 8) [Figure 1c, d]. There was a significant increase in MPA when PRP treated with the P2Y<sub>1</sub> receptor antagonist MRS2500 (1  $\mu$ M) was stimulated with 5  $\mu$ M ADP at 33 °C compared to 37 °C (57 ± 7 vs. 41 ± 9%, P < 0.05, n = 8) [Figure 2a] and with combined treatment with oral clopidogrel and MRS2500 *ex vivo* (15 ± 4 vs. 7 ± 3%, P < 0.001, n = 8) [Figure 2b]. 10  $\mu$ M AZD6140 *ex vivo* in addition to per oral clopidogrelmarkedly reduced MPA after 5  $\mu$ M ADP with no significant difference in MPA at 33 °C compared to 37 °C [Figure 2c].

#### Collagen-, 5-HT-, epinephrine-, and U46619-stimulated platelet aggregation before and after clopidogrel treatment

There was no significant difference in MPA when PRP was stimulated with 2 µg/ml collagen without clopidogrel at 33°C compared to 37°C (79 ± 1 vs. 83 ± 2%, P = NS, n = 8) or after oral clopidogrel (71 ± 5 vs 71 ± 4%, P = NS, n = 8) [Figure 3a, b]. There was a significant increase in MPA levels with 10 µM 5-HT at 33°C compared to 37°C both without clopidogrel (9.5 ± 1.5 vs. 6.3 ± 1.8%, P < 0.001, n = 8) and after oral clopidogrel (9.6 ± 2.4 vs. 6.0 ± 2.2%, P < 0.001, n = 8) [Figure 3c, d]. No differences in MPA between temperatures were observed with epinephrine 10 µM without clopidogrel (73 ± 6 vs 69 ± 7%, P = NS, n = 8) or after clopidogrel (61 ± 8 vs. 62 ± 7%, P = NS, n = 8) [Figure 4a, b] or with U46619 10 µM without clopidogrel (27 ± 14 vs 38 ± 14%, P = NS, n = 8) [Figure 4c, d].

#### Inhibition of P2Y<sub>12</sub> receptors evaluated by VASPphosphorylation

PRI (%) as assessed by the VASP kit was similar at 33 °C compared to 37 °C at baseline (71 ± 3 vs. 65 ± 7%, P = NS, n = 8) and after a 600 mg loading dose of clopidogrel (30 ± 7 vs. 27 ± 5%, P = NS, n = 8) [Figure 5a, b]. PRI (%) VASP was also similar at 33 °C compared to 37 °C with ADP and 10  $\mu$ M AZD6140 added *ex vivo* [Figure 5c].

### Dose response for ADP-stimulated platelet aggregation evaluated by LTA

To further evaluate the mechanism of increased sensitivity to ADP during hypothermia, we performed a narrow dose response curve for ADP-induced platelet aggregation in a new subset of healthy individuals. There was no significant increase in the plateau phase of MPA at 33 °C compared to 37°C, 72.0% (95% confidence interval (CI) 66.9–77.2) vs 77.8%, CI 66.9–88.6), P = NS, n = 7) [Figure 6]. However, there was leftward shift of the dose response curve indicating increased potency for ADP during hypothermia ( $EC_{50} = 0.68 \mu$ M (confidence interval 0.41–0.93)) compared to normothermia ( $EC_{50} = 1.38 \mu$ M (confidence interval 1.06–1.70)). MPA was significantly higher during hypothermia for 0.5, 1.0, and 1.5  $\mu$ M of ADP.

#### Discussion

We examined platelet reactivity before and after clopidogrel treatment at 37°C and at 33°C, a temperature used for the treatment of patients resuscitated after cardiac arrest. Mild hypothermia did not attenuate platelet aggregation. Instead, collagen, 5-HT, epinephrine, the thromboxane analogue U46619 and ADP-induced platelet aggregation was unaltered or even increased by hypothermia. Doseresponse curves revealed increased potency of ADP during hypothermia with no change in efficacy (maximum effect) compared with normothermia. Furthermore, the inhibitory effect of clopidogrel was attenuated. Similar attenuation of effect on ADP-induced aggregation was also seen after inhibition with the P2Y1 antagonist MRS2500. The decreased responsiveness to clopidogrel during hypothermia could be overcome by addition of the P2Y<sub>12</sub> antagonist AZD6140.

Dual antiplatelet therapy with aspirin and clopidogrel is recommended treatment for patients with acute coronary syndromes and after stent implantation [3]. A more rapid onset and higher level of platelet inhibition can be obtained with a 600 mg loading dose of clopidogrel, a dose recently endorsed for acute coronary syndromes to obtain better protection during percutaneous coronary interventions. A large number of patients with cardiac arrest have an acute coronary syndrome; some of them are treated with direct-PCI and stenting because of ST-elevation myocardial infarction, and therefore receive clopidogrel treatment. Despite this, the effect of clopidogrel has to our knowledge never been examined during mild hypothermia.

There is a general belief that mild hypothermia attenuates platelet aggregation [6,12], and it was somewhat surprising to find that collagen, 5-HT, epinephrine, the thromboxane analogue U46619 and ADP-stimulated platelet aggregation was unaltered or even increased by mild hypothermia. Most of the previous studies have been performed at lower temperatures, eg, 28°, 22°, or even 2°C [6,7,13]. Our interest was in the growing use of mild hypothermia (33–35°C) for the treatment of cardiac arrest, and perhaps in the future to reduce infarct size in patients with acute myocardial infarction. One study demonstrated a reduction in ADP-stimulated platelet



#### Figure I

AĎP-stimulated platelet aggregation at 5 and 20 μM [a, b] and after treatment with clopidogrel (600 mg) po [c, d]. n = 8. filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant, \*\*\* = P < 0.001.



Figure 2

ADP (5  $\mu$ M) -stimulated platelet aggregation after pretreatment with MRS2500 (1  $\mu$ M) ex vivo [a], after pretreatment with clopidogrel (600 mg) po and MRS2500 ex vivo [b], and after pretreatment with clopidogrel (600 mg) po and AZD6140 (10 µM) ex vivo [c]. n = 8. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant, \* = P < 0.05, \*\*\* = P < 0.001.

aggregation by 16% at 32°C compared to 37°C [5]. In contrast, a recent study by Heptinstall et al. demonstrated increased ADP-stimulated platelet aggregation at 28°C [9]. Lindenblatt et al[8] found increased ex vivo stimulated GpIIb/IIIa expression after TRAP stimulation at 34°C compared to normothermia, as well as accelerated thrombus formation in vivo in mice at 34°C. Our findings are also in agreement with Scharbert and co-workers[10], who found that platelet aggregation was increased with ADP, but unaltered with collagen during hypothermia. Further, shear stress-induced aggregation has been shown to increase at 32°C and 35°C[11].

In the present study, we found an attenuated effect of clopidogrel on ADP-stimulated platelet aggregation during mild hypothermia. The effect was highly significant and consistent at both doses of ADP used. This is in agreement with previous studies that have shown increased ADP responses during hypothermia in the presence of aspirin[9,10]. However, mild hypothermia has also been shown to augment the inhibitory effect of the reversible GpIIb/IIIa-blockers eptifibatide and tirofiban (but not that of the irreversible blocker abciximab)[5]. It could be hypothesized that hypothermia affects different classes of

platelet inhibitors differently. Such information is of course important in the selection of adequate platelet inhibition for patients treated with mild hypothermia.

Since the first report of a variable response to clopidogrel [14], a large number of studies have found a high prevalence of patients with pharmacodynamic poor responsiveness to clopidogrel [15]. Our data indicate that hypothermia induces a situation in which the effect of clopidogrel is reduced. The decreased responsiveness to clopidogrel during hypothermia could be overcome by addition of the reversible P2Y<sub>12</sub> antagonist AZD6140.

ADP activates two receptors on the platelet: the Gi-coupled P2Y<sub>12</sub> receptor coupled to inhibition of cAMP; and the Gq-coupled P2Y1 receptor, which stimulates IP3 and increases intracellular Ca2+. We tried to elucidate the mechanisms of the platelet effects induced by hypothermia by testing whether they were P2Y12-specific. We added the selective P2Y1-receptor antagonist MRS2500. Even in this situation, increased ADP sensitivity was seen during mild hypothermia. The effect was even more pronounced when MRS2500 was combined with clopidogrel.



#### Figure 3

Platelet aggregation stimulated by collagen (2  $\mu$ g/ml) [a, b] and by 5-HT (10  $\mu$ M) [c, d] before and after pre**treatement with clopidogrel (600 mg) po.** n = 8. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant, \*\*\* = p < 0.001.



#### Figure 4

 $Pi_{a}^{A}$ telet aggregation stimulated by epinephrine (10  $\mu$ M) [a, b] and U46619 (10  $\mu$ M) [c, d] before and after pretreatement with clopidogrel (600 mg) po. N = 6-8. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), n.s. = not significant.





ADP after clopidogrel p.o.







#### Figure 5

Flow cytometry study in which phosphorylation of VASP (and hence inhibition of platelet activation) is stimulated by PGE<sub>1</sub> and dephosphorylation (and hence augmentation of platelet activation) is mediated by ADP. Platelet activation is represented as platelet reactivity index (%PRI). ADP-stimulated platelet aggregation at baseline [a], after pretreatment with clopidogrel po [b], and after pretreatment with AZD6140 (10  $\mu$ M) ex vivo [c]. n = 8. Filled symbols represents hypothermia (33°), n.s. = not significant.

Thus, the increased platelet stimulatory effect of ADP can be seen regardless of which ADP receptor is blocked.

ADP activation of the  $P2Y_{12}$  receptor results in reduction of cAMP. VASP is an intracellular actin regulatory protein [16,17] that is phosphorylated into its P-VASP form by cAMP dependent protein kinases. ADP, the natural agonist to the  $P2Y_{12}$  receptor, inhibits VASP phosphorylation through inhibition of adenylate cyclase and downregulation of cAMP production. We examined if the mechanism of the platelet effects of hypothermia are mediated at the level of VASP-phosphorylation. However, we saw no effect of hypothermia on VASP-phosphorylation when whole blood was stimulated by ADP alone or in combination with clopidogrel or AZD6140.

The mechanism of the increased ADP-induced platelet aggregation during hypothermia observed in our study remains elusive. It does not seem to depend on antagonist-receptor interaction or intracellular second messenger systems. Another possible explanation would be altered degradation rates of ADP. However, we repeated the experiments with the stable ADP analogue 2-MeSADP and obtained similar results (data not shown). To understand the receptor pharmacology during hypothermia, we assessed dose-response curves for ADP. Platelets at 33°C displayed an increased sensitivity to ADP and epinephrine compared to 37°C, with a leftward shift of the doseresponse curve and a significantly lower EC50 value for platelets at 33°C. Thus, it was only possible to see a difference in effect when doses in the sigmoidal part of the curve were tested, with the maximum effect being unaltered compared with normothermia. This observation may explain some of the conflicting data in the literature. It also indicates that it is important to take the concentration of the agonist into consideration when studying hypothermia and platelets.

Haemostasis during hypothermia is complex, and it is possible that platelet responses differ at different temperatures. We found unaltered or even increased platelet



Figure 6

ADP dose response curve for platelet aggregation at normothermia and hypothermia. Filled symbols represents normothermia (37°), open symbols represents hypothermia (33°), \* = p < 0.05, \*\* = p < 0.05, \*\* = p < 0.001.

responses during mild hypothermia. The literature contains conflicting data, but in support of our findings, increased platelet activation has been seen during profound hypothermia[13] and increased aggregation has been reported at a more intermediate temperature of 28°C [9]. However, prolongation of bleeding time has been reported in clinical situations with hypothermia [18-20]. It is possible that this primarily reflects effects of hypothermia on the coagulation system, especially a decrease in the fibrinolysis-inhibiting  $\alpha_2$ -macroglobulin levels [21]. On the other hand, unintentional perioperative hypothermia is associated with postoperative myocardial ischemia, indicating a prothrombotic effect of hypothermia, and platelet count and platelet activity have been shown to be increased in this setting [12,21].

#### Conclusion

In conclusion, our study indicates that platelet reactivity is unaltered and in some situations increased during mild hypothermia. The inhibitory effect of clopidogrel was attenuated in our study. The clinical conclusion is that we cannot rely on hypothermia per se as a platelet inhibitor. Based on current *ex vivo* evidence, dual platelet inhibitor with aspirin and clopidogrel is probably needed for patients with acute coronary syndromes treated with mild hypothermia. Since hypothermia induces a state of reduced clopidogrel responsiveness, it is possible that new reversible ADP blockers such as AZD6140 could be beneficial. Clinical studies are needed to determine the best use of platelet and coagulation inhibitors for the growing number of patients treated with mild hypothermia.

#### **Competing interests**

The authors declare that they have no competing interests.

#### **Authors' contributions**

CH carried out the platelet function studies, analyzed and participated in writing the manuscript. DE and OB designed the study, analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

#### Acknowledgements

The study has been supported by the Swedish Heart and Lung Foundation, Swedish Scientific Research Council, The Vascular Wall Program, and Lund University. David Erlinge is a holder of The Lars Werkö distinguished research fellowship from the Swedish Heart and Lung Foundation.

#### References

- Bernard SA, Gray TW, Buist MD, Jones BM, Silvester W, Gutteridge G, Smith K: Treatment of comatose survivors of out-of-hospital cardiac arrest with induced hypothermia. N Engl J Med 2002, 346:557-63.
- Hypothermia after Cardiac Arrest Study Group: Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. N Engl J Med 2002, 346:549-56.
- 3. Braunwald E, Antman EM, Beasley JW, Califf RM, Cheitlin MD, Hochman JS, Jones RH, Kereiakes D, Kupersmith J, Levin TN, Pepine G, Schaeffer JW, Smith EE 3rd, Steward DE, Theroux P, Gibbons RJ, Alpert JS, Faxon DP, Fuster V, Gregoratos G, Hiratzka LF, Jacobs AK, Smith SC Jr, American College of Cardiology/American Heart Association Task Force on Practice G: ACC/AHA guideline update for the management of patients with unstable angina and non-ST-segment elevation myocardial infarction-2002: summary article: a report of the American College of Cardiology/ American Heart Association Task Force on Practice Guidelines (Committee on the Management of Patients With Unstable Angina). Circulation 2002, 106:1873-900.
- Dae MW, Gao DW, Sessler DI, Chair K, Stillson CA: Effect of endovascular cooling on myocardial temperature, infarct size, and cardiac output in human-sized pigs. Am J Physiol Heart Grc Physiol 2002, 282:H1584-91.
- Frelinger AL 3rd, Furman MI, Barnard MR, Krueger LA, Dae MW, Michelson AD: Combined effects of mild hypothermia and glycoprotein IIb/IIIa antagonists on platelet-platelet and leukocyte-platelet aggregation. Am J Cardiol 2003, 92:1099-101.
- Michelson AD, MacGregor H, Barnard MR, Kestin AS, Rohrer MJ, Valeri CR: Reversible inhibition of human platelet activation by hypothermia in vivo and in vitro. Thromb Haemost 1994, 71:633-40.
- Michelson AD, Barnard MR, Khuri SF, Rohrer MJ, MacGregor H, Valeri CR: The effects of aspirin and hypothermia on platelet function in vivo. Br J Haematol 1999, 104:64-8.
- Lindenblatt N, Menger MD, Klar E, Vollmar B: Sustained hypothermia accelerates microvascular thrombus formation in mice. *Am J Physiol Heatt Circ Physiol* 2005, 289:142680-7.
- Xavier RG, White AE, Fóx SC, Wilcox RG, Heptinstall S: Enhanced platelet aggregation and activation under conditions of hypothermia. Thromb Haemost 2007, 98:1266-75.
   Scharbert G, Kalb M, Marschalek C, Kozek-Langenecker SA: The
- Scharbert G, Kalb M, Marschalek C, Kozek-Langenecker SA: The effects of test temperature and storage temperature on platelet aggregation: a whole blood in vitro study. Anesth Analg 2006, 102:1280-4.
- Zhang JN, Wood J, Bergeron AL, McBride L, Ball C, Yu Q, Pusiteri AE, Holcomb JB, Dong JF: Effects of low temperature on shearinduced platelet aggregation and activation. J Trauma 2004, 57:216-23.
- Faraday N, Rosenfeld BA: In vitro hypothermia enhances platelet GPIIb-IIIa activation and P-selectin expression. Anesthesiology 1998, 88:1579-85.
- White JG, Krivit W: An ultrastructural basis for the shape changes induced in platelets by chilling. Blood 1967, 30:625-35.

#### Thrombosis Journal 2009, 7:2

- Jaremo P, Lindahl TL, Fransson SG, Richter A: Individual variations of platelet inhibition after loading doses of clopidogrel. J Intern Med 2002, 252:233-8.
- Bliden KP, DiChiara J, Tantry US, Bassi AK, Chaganti SK, Gurbel PA: Increased risk in patients with high platelet aggregation receiving chronic clopidogrel therapy undergoing percutaneous coronary intervention: is the current antiplatelet therapy adequate? J Am Coll Cardiol 2007, 49:657-66.
   Reinhard M, Giehl K, Abel K, Haffner C, Jarchau T, Hoppe V, Jockusch
- Reinhard M, Giehl K, Abel K, Haffner C, Jarchau T, Hoppe V, Jockusch BM, Walter U: The proline-rich focal adhesion and microfilament protein VASP is a ligand for profilins. Embo J 1995, 14:1583-9.
- Halbrugge M, Friedrich C, Eigenthaler M, Schanzenbacher P, Walter U: Stoichiometric and reversible phosphorylation of a 46kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators. J Biol Chem 1990, 265:3088-93.
- Khuri SF, Wolfe JA, Josa M, Axford TC, Szymanski I, Assousa S, Ragno G, Patel M, Silverman A, Park M, et al.: Hematologic changes during and after cardiopulmonary bypass and their relationship to the bleeding time and nonsurgical blood loss. J Thorac Cardiovacs Surg 1992, 104:94-107.
   Patt A, McCroskey BL, Moore EE: Hypothermia-induced coagu-
- Patt A, McCroskey BL, Moore EE: Hypothermia-induced coagulopathies in trauma. Surg Clin North Am 1988, 68:775-85.
   Schmied H, Kurz A, Sessler DI, Kozek S, Reiter A: Mild hypother-
- Schmied H, Kurz A, Sessler DJ, Kozek S, Reiter A: Mild hypothermia increases blood loss and transfusion requirements during total hip arthroplasty. Lancet 1996, 347:289-92.
- Elwood PC, Beswick A, O'Brien JR, Renaud S, Fifield R, Limb ES, Bainton D: Temperature and risk factors for ischaemic heart disease in the Caerphilly prospective study. Br Heart J 1993, 70:520-3.



Page 10 of 10 (page number not for citation purposes)

# Paper II


International Journal of Cardiology 142 (2010) 187-192



www.elsevier.com/locate/ijcard

## The reversible oral P2Y<sub>12</sub> antagonist AZD6140 inhibits ADP-induced contractions in murine and human vasculature

Carl Högberg<sup>a</sup>, Helen Svensson<sup>a</sup>, Ronny Gustafsson<sup>b</sup>, Atli Eyjolfsson<sup>b</sup>, David Erlinge<sup>a,\*</sup>

<sup>a</sup> Department of Cardiology, Lund University Hospital, SE-221 85, Lund, Sweden <sup>b</sup> Cardiothoracic Surgery, Lund University Hospital, SE-221 85, Lund, Sweden

> Received 15 September 2008; accepted 13 December 2008 Available online 26 January 2009

#### Abstract

*Objectives:* The platelet ADP P2Y<sub>12</sub> receptor which is a target for the antithrombotic drug clopidogrel is also distributed on vascular smooth muscle cells and stimulate contraction. This study investigates whether AZD6140, in contrast to clopidogrel, can inhibit ADP-mediated arterial contractions. *Methods:* Mice were treated with clopidogrel, 50 mg/kg, 24 and 2 h before experiment. Thoracic aorta ring segments from both clopidogrel-treated (n=5) and untreated (n=4) mice were mounted in myograph baths. Contractions of human left internal mammary arteries (IMA) and small arteries were studied in an identical manner.

*Results:* Clopidogrel treatment per os did not inhibit contractions by the stable ADP analogue 2-MeSADP (10  $\mu$ M). However, addition of 1  $\mu$ M AZD6140 in vitro inhibited ADP contraction (% of maximal contraction by 60 mM K<sup>+</sup>) both in the clopidogrel-treated, from 64% to 32% (*P*=0.002) and in the untreated group, from 59% to 33% (*P*=0.015). 2-MeSADP contractions in human IMA and small arteries were inhibited by AZD6140.

*Conclusions:* The antiplatelet drug AZD6140 blocks the contractile effects of ADP in both murine and human vasculature. These effects of AZD6140 could be beneficial in the management of conditions in which vasospasm may play a role.

© 2008 Elsevier Ireland Ltd. All rights reserved.

Keywords: Vasoconstriction; Receptors; Platelets

#### 1. Introduction

The platelet P2Y<sub>12</sub> receptor is an established target for inhibiting ADP-mediated platelet aggregation, thus preventing thrombosis. The thienopyridine ticlopidine has been shown to prevent stent thrombosis, and the second-generation thienopyridine clopidogrel to reduce the incidence of myocardial infarction in patients with acute coronary syndromes (ACS) (CURE) and mortality in patients with ST-elevation myocardial infarction (COMMIT) [1,2]. The third-generation thienopyridine prasugrel is associated with significantly reduced rates of ischaemic events, including stent thrombosis in ACS patients with scheduled percutaneous coronary intervention, compared to clopidogrel (TRITON) [3].

The thienopyridines ticlopidine, clopidogrel, and prasugrel are prodrugs with bioactive metabolites that reduce platelet aggregation by irreversibly binding to the  $P2Y_{12}$ receptor [4]. Irreversible binding to the  $P2Y_{12}$  receptor is a problem in patients in need of acute coronary bypass surgery, other invasive procedures, or when bleeding complications occur. To regain  $P2Y_{12}$  receptor function, platelets must be regenerated either by transfusion or through the natural platelet life cycle of approximately 10 days.

AZD6140 is a new, orally active cyclopentyl-triazalopyramidine (CPTP) that does not require metabolic activation and inhibits platelet aggregation via reversible binding to the P2Y<sub>12</sub> receptor. It is currently being studied for its potential to prevent thrombotic events in patients with ACS [5]. In clinical studies to date, AZD6140 showed greater and

<sup>\*</sup> Corresponding author. Tel.: +46 46 17 25 97; fax: +46 46 15 78 57. *E-mail address:* david.erlinge@med.lu.se (D. Erlinge).

<sup>0167-5273/\$ -</sup> see front matter © 2008 Elsevier Ireland Ltd. All rights reserved. doi:10.1016/j.ijcard.2008.12.091

more consistent inhibition of platelet aggregation than clopidogrel, with a similar incidence of total bleeding events [6]. Due to its reversible antagonistic effect, discontinuation of AZD6140 results in rapid return of ADP  $P2Y_{12}$ -receptor function as drug plasma levels fall [7].

 $P2Y_{12}$  receptors are also found on vascular smooth muscle cells (VSMC), where they mediate arterial contraction after stimulation by ADP [8]. However, the contractions are not inhibited in blood vessels obtained from patients treated with clopidogrel [8], perhaps because the agent's active metabolite reaches low systemic concentrations and has a short half-life [9]. Furthermore, even the clopidogrel effects on platelets are variable between patients and clopidogrel "resistance" is common [9]. We therefore wanted to repeat the experiments in an animal model were we could maximize the clopidogrel dose to see if inhibition of ADP contractions could be achieved.

A short peak of high systemic levels of the active metabolite could result in an irreversible blockade of the  $P2Y_{12}$  receptors on the VSMC, but the effect is not expected to be as permanent as in platelets, since VSMC constantly synthesize new  $P2Y_{12}$  receptors. In contrast to clopidogrel, AZD6140 reaches continuous high systemic concentrations and has the potential to block vascular  $P2Y_{12}$  receptors. It could therefore have beneficial effects not only by inhibiting platelet aggregation, but also by preventing vasospasm.

The aim of this study was: (1) To evaluate if clopidogrel administered in high doses (1000-fold higher per kg compared to regular clinical doses) could inhibit P2Y<sub>12</sub>-receptor mediated contractions in a mouse model. (2) To evaluate the effect of AZD6140 on P2Y<sub>12</sub>-mediated contraction in mice in the presence and absence of orally administered clopidogrel. (3) To examine if AZD6140 could inhibit ADP-mediated contraction in human internal mammary arteries and small human arteries.

#### 2. Methods

#### 2.1. Mouse experiments

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Five of 9 female mice were pretreated with clopidogrel 50 mg/kg the day before and 2 h before sacrifice and tissue harvest; 4 mice received no clopidogrel treatment. Thoracic aorta sections taken from all mice were dissected from connective tissue and denuded by gently rubbing the intimal surface with a wooden stick. Ring segments (7 or 8 per mouse) were mounted into temperature-controlled tissue baths with Krebs buffer (mM): NaCl 119, NaHCO<sub>3</sub> 15, KCI 4.6, MgCl<sub>2</sub> 1.2, NaH2PO4 1.2, CaCl<sub>2</sub> 1.5, and glucose 5.5.

#### 2.2. Human blood vessels

The investigation conforms to the principles outlined in the Declaration of Helsinki [10]. Internal mammary artery

segments and pericardial fat arteries were obtained from 9 patients undergoing coronary bypass surgery (mean age=72 years), 7 were men and 3 had non-insulin-dependent diabetes mellitus. The blood vessels were put in cold buffer solution (see below) and transported to the laboratory where they were dissected free of adhering tissue under a microscope. The vessels were immediately used for experiments. The internal mammary arteries had a relaxed inner diameter of approximately 1 mm while the pericardial fat arteries had a relaxed inner diameter of approximately 0.3-0.5 mm. The endothelial layer of the internal mammary arteries was removed by gently rubbing the intimal surface with a wooden stick before mounting it in the myograph. The endothelium of the pericardial fat arteries was removed by perfusion with TritonX (0.1%, 10 s), followed by perfusion with buffer solution. This was confirmed by demonstrating a lack of dilatory effects of 1 uM acetylcholine.

#### 2.3. Myograph experiments

The pharmacological responses were analyzed using tissue baths for measurement of isometric tension. The experimental setting has previously been described [11,12]. Mouse aorta and the small human arteries were precontracted to 1 mN and human internal mammary arteries to 2 mN. The isometric tension in each segment was continuously recorded, and the maximum contractile capacity of the segments was determined by exposure to 60 mM K<sup>+</sup>. AZD6140 1 µM or dimethyl sulfoxide (DMSO) 1:1000 as control was added. After 20 min, segments were slightly precontracted with 10 nM norepinephrine (NE) and then further contracted with 10 µM of the stable ADP molecule 2-methylthio-ADP (2-MeSADP). Results are presented as percent of K<sup>+</sup> induced contraction. To study the response to 2-MeSADP without interference of simultaneous P2X1-receptor-induced responses, all vessels were desensitized with 10  $\mu$ M  $\alpha$ , $\beta$ -methylene ATP ( $\alpha\beta$ -MeATP) for 10 min as described earlier [13].

The experiments were conducted as follows: first, the potassium-induced contraction was tested (and found to be similar between all compared groups). After 1.5 h of resting, the antagonist (AZD6140) or control saline solution with DMSO 1:1000 was added. AZD6140 did not have any effect on resting tone in any of the examined blood vessels. The arteries were then stimulated with 1  $\mu$ M of  $\alpha\beta$ -MeATP, which stimulates the ATP receptor P2X<sub>1</sub>, an ion-channel receptor that desensitizes very quickly. Within 5 min the contraction was back at baseline. Norepinephrine was then added to produce a small precontraction, After 10 min, when the NE contraction had stabilised, 2-MeSADP was added. The 2-MeSADP-induced contractions were calculated with-out including any remaining NE contraction.

### 2.4. Drugs

 $\alpha\beta$ -MeATP, 2-MeSADP, and NE were purchased from Sigma Co., USA. Clopidogrel was purchased from Sequoia

Research Products, Ltd., Oxford, UK. AZD6140 was provided by AstraZeneca, Mölndal, Sweden. All drugs were dissolved in 0.9% saline, except AZD6140 and its control saline solution, which were dissolved in DMSO 1:1000. PCR consumables were purchased from Life



Technologies, Inc., USA, or Perkin–Elmer Applied Biosystems Inc, Foster City, CA, USA.

### 2.5. Ethics

The Ethics Committee of Lund University approved the project. All patients submitted written consent to participate in the study. The study conforms with US National Institutes of Health guidelines, and the Declaration of Helsinki.

#### 2.6. Calculation and statistics

Calculations and statistics were performed using Graph-Pad Prism 4.0 software. Contraction was calculated as the percentage of the contraction with 60 mM K<sup>+</sup>. Statistical significance was accepted when P<0.05, using Student's *t*test analysing the 2-MeSADP data. Values are presented as means±SEM. *n*=number of vessel segments.

#### 3. Results

#### 3.1. The effect of AZD6140 on mouse aorta

10  $\mu$ M 2-MeSADP induced prominent contractions in mouse aorta of 59±7% (measured as % of maximal contraction induced by 60 mM K<sup>+</sup>) from otherwise untreated mice. The contractions were significantly reduced by 1  $\mu$ M of the P2Y<sub>12</sub> antagonist AZD6140 to 33±6% (*P*=0.015 vs. clopidogrel, *n*=9 vessel segments per group from 4 resp 5 mice, Fig. 1A). From mice treated with oral clopidogrel, the 2-MeSADP-induced contractions in mouse aorta were similar to those from untreated mice (*P*=n.s., *n*=5 vessel segments per group from 4 resp 5 mice, Fig. 1B). Even in aorta from mice pretreated with oral clopidogrel, did 1  $\mu$ M of the P2Y<sub>12</sub> antagonist AZD6140 significantly inhibit the contractions induced by 2-MeSADP: 64±8% vs. 32±6% (*P*=0.002, *n*=17 vessel segments per group from 4 resp 5 mice, Fig. 1C).

### 3.2. The effect of AZD6140 on human internal mammary artery

10  $\mu$ M 2-MeSADP induced prominent contractions in human internal mammary artery, as shown in an earlier study [8]. The contractions were significantly reduced by 1  $\mu$ M of

Fig. 1. (A) AZD6140 1  $\mu$ M significantly inhibits the contractile response in mouse aorta to a single dose of 2-MeSADP 10  $\mu$ M. DMSO 1:1000 was added in control (n=9 vessel segments per group from 4 resp. 5 mice, see "Methods"). (B) Oral clopidogrel 50 mg/kg din ot have any effect on contractile response in mouse aorta to a single dose of 2-MeSADP 10  $\mu$ M (n=5 vessel segments per group, from 5 resp. 4 mice) (C) AZD6140 1  $\mu$ M significantly inhibits the contractile response in mouse aorta to a single dose of 2-MeSADP 10  $\mu$ M (n=5 vessel segments per group, from 5 resp. 4 mice) (C) AZD6140 1  $\mu$ M significantly inhibits the contractile response in mouse aorta to a single dose of 2-MeSADP 10  $\mu$ M even in the presence of oral clopidogrel treatment 50 mg/kg. (n=17 vessel segments per group from 5 resp. 4 mice) The contractions are expressed as percentage of an initial contraction induced by 60 mM K<sup>+</sup>. Data are shown as means±SEM, \*P<0.015, \*\*P=0.002. The vessel segments were taken from 5 clopidogrel-treated and 4 untreated mice.



Fig. 2. AZD6140 inhibits ADP-induced contractions in both (A) human internal mammary arteries (n=15 vessel segments per group from 7 patients). And (B) small arteries (n=10 vessel segments per group from 7 patients). Contractile response in human internal mammary artery and small arteries to a single dose of 10  $\mu$ M 2-MeSADP (see "Methods"). The contractions are expressed as percentage of an initial contraction induced by 60 mM K<sup>+</sup>. Data are shown as means ±SEM. \*P<0.05, \*\*P<0.01.

AZD6140, from  $52\pm10\%$  to  $29\pm7\%$  (*P*<0.05, *n*=15 vessel segments per group from 7 patients, Fig. 2A).

### 3.3. The effect of AZD6140 on human small pericardial fat arteries

2-MeSADP induced contractions in human small pericardial fat arteries. The contractions were significantly reduced by 1  $\mu$ M of AZD6140, from 12±3% to 2±1% (*P*<0.01, *n*=10 vessel segments per group from 7 patients, Fig. 2B). 3.4. The effect of AZD6140 on contractions by other receptor agonists

10  $\mu M$  norepinephrine was added to produce a precontraction, which gave a maximum contraction of  $30\pm5\%$  in the internal mammary artery and  $10\pm3\%$  in pericardial fat arteries. AZD6140 1  $\mu M$  did not have any effect on the NE-induced contractions in either internal mammary artery or pericardial fat arteries (Fig. 3). Similarly, AZD6140 1  $\mu M$  did not have any effect on the  $\alpha\beta$ -MeATP-induced contractions in either internal mammary artery or pericardial fat arteries (Fig. 4). A similar lack of effect of AZD6140 on NE and  $\alpha\beta$ -MeATP stimulated contractions were found in mouse aorta (data not shown).

### 4. Discussion

We have previously shown that the ADP P2Y<sub>12</sub> receptor mediates vasoconstriction in human internal mammary artery and subcutaneous vein [8]. Here we demonstrate ADPstimulated, P2Y<sub>12</sub>-mediated contractions in the mouse aorta. High doses of oral clopidogrel treatment had no inhibitory effect, while AZD6140 added *ex vivo* inhibited P2Y<sub>12</sub>-mediated vasoconstriction in murine vessels regardless of clopidogrel



Fig. 3. Examination of specificity of AZD6140: contractile response in (A) human internal mammary artery (n=15 per group) and (B) small arteries (n=11 per group) to 10 nM norepinephrine (NE). There was no effect of AZD6140 on NE-induced vasoconstriction in either of the arteries. The contractions are expressed as percentage of an initial contraction induced by 60 mM K<sup>+</sup>. Data are shown as means±SEM.



Fig. 4. Examination of specificity of AZD6140. Contractile response in (A) human internal mammary artery (n=8 per group) and (B) small arteries (n=11 per group) to 1  $\mu$ M AZD6140. There was no effect of AZD6140 on  $\alpha\beta$ -MeATP-induced P2X1-mediated vasoconstriction in either of the arteries. The contractions are expressed as percentage of an initial contraction induced by 60 mM K<sup>+</sup>. Data are shown as means±SEM.

pretreatment. Furthermore, AZD6140 blocked ADP-induced vasoconstrictions in both large and small human arteries.

The finding of an effect of ADP both on platelet aggregation and on arterial contraction is not surprising. Several other platelet aggregation factors are also stimulators of vasoconstriction, e.g., epinephrine, thromboxane, and serotonin. The combined effect could be an evolutionary adaptation important in stopping bleeding. Previously, a contractile effect of ADP acting on  $P2Y_{12}$  receptors in arteries had only been demonstrated in human blood vessels [8]. Here we demonstrate  $P2Y_{12}$ -mediated vasoconstrictions in another species, the mouse. Thus,  $P2Y_{12}$ -mediated vasoconstriction may be a general phenomenon.

The competitive  $P2Y_{12}$  antagonist AZD6140 significantly inhibited the contractions in mouse aorta, demonstrating a potential new physiological effect of the drug, in addition to its inhibitory effects on platelet aggregation. As demonstrated in human blood vessels [8], oral clopidogrel treatment did not block  $P2Y_{12}$ -receptor-mediated vasoconstriction. If the active metabolite of clopidogrel reaches the artery, it should remain irreversibly bound to the receptor during the short time between removal of the aorta and the contraction experiments. However, even in the present study, high oral clopidogrel doses given to mice did not result in an inhibitory effect in murine vasculature (Fig. 2B). This is probably due to low systemic concentrations of the active metabolite of clopidogrel [9].

Clopidogrel could not be added *in vitro* because it is a prodrug. AZD6140 per os was not tested because it is a reversible drug and is expected to be washed away during dissection. AZD6140 blocked ADP-induced vasoconstriction mediated by P2Y<sub>12</sub> receptors in denuded mouse aortic rings regardless of oral pretreatment with clopidogrel. The effect was similar as in the absence of clopidogrel (Fig. 2).

In large arteries of patients with ACS, the high concentrations of ADP released in a platelet thrombus could contribute to the vasospasm often seen in a culprit lesion. The vasospasm worsens the ischemia caused by the thrombus. We therefore examined the effect of AZD6140 on the left internal mammary artery. AZD6140 significantly inhibited the ADP-mediated contractions in human left internal mammary artery. Since AZD6140 reaches therapeutic serum concentration *in vivo*, it is possible that it may have a dual anti-ischemic effect in patients by inhibiting both thrombus formation and vasospasm, although any clinically significant benefit would need to be validated in a prospective clinical trial.

We also examined the effect of ADP in small arteries, approximately 0.3-0.5 mm in internal diameter, which are involved in blood flow regulation. The inhibitory effects of AZD6140 were even more prominent in these smaller arteries, indicating a possible role in regulating tissue perfusion. The net effect of ADP is a balance between its vasodilatory effects via endothelial activation and contractile effects on the smooth muscle cells. The contractile effects of ADP have previously been masked by the vasodilatory effect of ADP mediated via activation of P2Y<sub>1</sub> receptors on the endothelium [14,15], and endothelial denudation was a prerequisite in our experiments to reveal the contractile effects. ADP mediated vasoconstriction is most likely of importance in situations of endothelial damage, such as in a ruptured plaque.

AZD6140 did not have any effect on NE-induced contractions ( $\alpha_1$ -receptors) or contractions mediated by the ATP receptor P2X<sub>1</sub> in either murine or human vessels, indicating a specific effect on P2Y<sub>12</sub> receptors. This is in agreement with previous studies in platelets [5].

Interestingly, nicotine has been shown to prominently upregulate  $P2Y_{12}$  receptors in human VSMC [16]. This nicotinic upregulation of  $P2Y_{12}$  is mediated by the nicotinic acetylcholine receptors. Studies in platelets have shown that the functional and active  $P2Y_{12}$  receptor is organised as homooligomeric complexes [17], represented in cholesterol dependent microdomains called lipid rafts found in the cell membranes [18]. We speculate that high cholesterol and nicotine may have an upregulating effect on the  $P2Y_{12}$  receptor, and hence the risk of atherothrombosis.

In conclusion, clopidogrel does not block ADP mediated contraction, not even when given in high doses. AZD6140 inhibits ADP-mediated vascular contraction both in murine and human vasculature. The effects of AZD6140 could be beneficial in the management of conditions in which vasospasms may play a role.

### Funding

The study has been supported by the Swedish Heart and Lung Foundation, Swedish Scientific Research Council, the Zoegas Foundation, The Vascular Wall Program, and Lund University. David Erlinge is a holder of The Lars Werkö distinguished research fellowship from the Swedish Heart and Lung Foundation.

### Conflict of interest

The authors received an institutional research grant from AstraZeneca to conduct this research.

#### Acknowledgement

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology [19].

#### References

- [1] Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med 2001;345:494–502.
- [2] Chen ZM, Jiang LX, Chen YP, et al. Addition of clopidogrel to aspirin in 45,852 patients with acute myocardial infarction: randomised placebo-controlled trial. Lancet 2005;366:1607–21.
- [3] Wiviott SD, Braunwald E, McCabe CH, et al. Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med 2007;357:2001–15.
- [4] Gachet C, Hechler B. The platelet P2 receptors in thrombosis. Semin Thromb Hemost 2005;31:162–7.
- [5] van Giezen JJ, Humphries RG. Preclinical and clinical studies with selective reversible direct P2Y12 antagonists. Semin Thromb Hemost 2005;31:195–204.

- [6] Husted SE, Ziegler BK, Kher A. Long-term anticoagulant therapy in patients with coronary artery disease. Eur Heart J 2006;27:913–9.
- [7] Cannon CP, Husted S, Harrington RA, et al. Safety, tolerability, and initial efficacy of AZD6140, the first reversible oral adenosine diphosphate receptor antagonist, compared with clopidogrel, in patients with non-STsegment elevation acute coronary syndrome: primary results of the DISPERSE-2 trial. J Am Coll Cardiol 2007;50:1844–51.
- [8] Wihlborg AK, Wang L, Braun OO, et al. ADP receptor P2Y12 is expressed in vascular smooth muscle cells and stimulates contraction in human blood vessels. Arterioscler Thromb Vasc Biol 2004;24:1810–5.
- [9] Wallentin L, Varenhorst C, James S, et al. Prasugrel achieves greater and faster P2Y12receptor-mediated platelet inhibition than clopidogrel due to more efficient generation of its active metabolite in aspirin-treated patients with coronary artery disease. Eur Heart J 2008;29:21–30.
- [10] World Medical Association Declaration of Helsinki. Recommendations guiding physicians in biomedical research involving human subjects. Cardiovasc Res 1997;35:2–4.
- [11] Wihlborg AK, Malmsjö M, Eyjolfsson A, Gustafsson R, Jacobson K, Erlinge D. Extracellular nucleotides induce vasodilatation in human arteries via prostaglandins, nitric oxide and endothelium-derived hyperpolarising factor. Br J Pharmacol 2003;138:1451–8.
- [12] Borna C, Wang L, Gudbjartsson T, et al. Contractions in human coronary bypass vessels stimulated by extracellular nucleotides. Ann Thorac Surg 2003;76:50–7.
- [13] Kasakov L, Burnstock G. The use of the slowly degradable analog, alpha, beta-methylene ATP, to produce desensitisation of the P2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. Eur J Pharmacol 1982;86:291–4.
- [14] Malmsjo M, Edvinsson L, Erlinge D. P2U-receptor mediated endothelium-dependent but nitric oxide-independent vascular relaxation. Br J Pharmacol 1998;123:719–29.
- [15] Burnstock G, Kennedy C. A dual function for adenosine 5'-triphosphate in the regulation of vascular tone. Excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. Circ Res 1986;58:319–30.
- [16] Shanker G, Kontos JL, Eckman DM, Wesley-Farrington D, Sane DC. Nicotine upregulates the expression of P2Y12 on vascular cells and megakaryoblasts. J Thromb Thrombolysis 2006;22:213–20.
- [17] Savi P, Zachayus JL, Delesque-Touchard N, et al. The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. Proc Natl Acad Sci U S A 2006;103:11069–74.
- [18] Simons K, Ehehalt R. Cholesterol, lipid rafts, and disease. J Clin Invest 2002;110:597–603.
- [19] Coats AJ. Ethical authorship and publishing. Int J Cardiol 2009;131: 149–50.

# Paper III

### Succinate independently stimulates full platelet activation via cAMP and PI3 $\beta$ kinase signaling

C. Högberg, O. Gidlöf, C. Tan, S. Svensson, J. Nilsson-Öhman, D. Erlinge, B. Olde\* *Department of Cardiology, Lund University Hospital, SE-221 84 Lund, Sweden* \*Corresponding author: Björn Olde, Ph.D,. BMC D12, 22184 Lund, Sweden. Telephone: +46 46 2227729 E-mail: bjorn.olde@med.lu.se

Journal of Thrombosis and Haemostasis (in press)

Summary. Background: The citric cycle intermediate succinate has recently been identified as ligand for the G-protein coupled receptor (GPCR) SUCNR1. We have previously found that this receptor is one of the most expressed GPCRs in human platelets. Objective: The aim of this study was to investigate the role of SUCNR1 in platelet aggregation and to explore the signalling pathways of this receptor in platelets. Methods and Results: Using RT-PCR, we could demonstrate that SUCNR1 is expressed in human platelets at a level corresponding to that of the P2Y1 receptor. Light transmission aggregation experiments showed a dose-dependent aggregation induced by succinate reaching a maximum response at 0.5mM. The effect of succinate on platelet aggregation was confirmed with flow cytometry showing increased surface expression of activated GPIIb/IIIa, and P-selectin. Intracellular SUCNR1 signalling was found to result in decreased cAMP levels, Akt phosphorylation mediated by PI3K $\beta$  activation and receptor desensitisation. Further, succinate-induced platelet aggregation was demonstrated to depend on Src, generation of thromboxane A<sub>2</sub> and ATP release. The platelet SUCNR1 is subject to desensitization through both homologous and heterologous mechanisms. In addition, the P2Y<sub>12</sub> receptor inhibitor ticagrelor completely prevented platelet aggregation induced by succinate. Conclusions: Our experiments show that succinate induces full aggregation of human platelets via SUCNR1. Succinate-induced platelet aggregation depends on thromboxane A2 generation, ATP release and P2Y12 activation.

Keywords: Succinate, SUCNR1, GPR91, platelet, PI3-Kinase, GPCR

### Introduction

The dicarboxyl acid succinate is known to be a physiologically important intermediate in the citric acid cycle where it plays a crucial role in mitochondrial energy production [1]. In recent years it has become clear that succinate also functions as ligand for the membrane receptor SUCNR1 [2]. The transcript for the human SUCNR1 receptor encodes a 330-amino acid protein that belongs to the genetic superfamily of G-protein coupled receptors (GPCR) [3]. SUCNR1 has been identified and quantified in many important organs throughout the human body particularly high levels of SUCNR1 are present in the spleen, liver, testis and kidneys [4-7]. When we

examined mRNA expression of GPCRs in platelets using microarray and realtime-PCR, the succinate receptor displayed the third highest expression of all GPCRs examined [7], indicating an important role in platelet regulation.

More than two decades ago Huang et al. [8] discovered that succinate had a potentiating effect on platelet activating substances like adenosine 5'-dihosphate (ADP). epinephrine and serotonin. Huang et al. [8] also concluded that high concentrations. 5 - 10 mM of succinate could induce primary platelet aggregation. The potentiating effect of succinate on ADP has also been described by Macaulay et al. [6]. Succinate normally is present systemically in concentrations that range between 1 – 20 µM [9], but during pathophysiological conditions like ischemia and metabolic stress, plasma levels of succinate have been reported to reach millimolar concentrations [10-12]

Succinate, is a multi-functional molecule with a physiological potential that has not yet been fully evaluated. The aim of this study is to characterize the platelet succinate receptor SUCNR1 signaling pathways and to determine the role of this novel receptor in the aggregation process. This involves investigating regulation and function of SUCNR1 in signaling pathways with known importance platelet function. Our experiments demonstrate that succinate alone is capable of mediating full platelet aggregation by decreasing cAMP levels and activating the PI3Kβ/Akt pathway leading to P-selectin and GPIIb/IIIa activation. Furthermore, SUCNR1 is dependent on Src kinase signaling and is subject to homologous as well as heterologous desensitization.

### Methods and material

### Chemicals

Sodium succinate dibasic hexahydrate, 2'-Deoxy-N6-methyladenosine 3'. 5'bisphosphate tetrasodium salt (MRS2179). Ethylenediaminetetraacetic acid disodium salt solution (EDTA), prostaglandin E1 (PGE1). Apyras. Forskolin, prostaglandin I<sub>2</sub> (PGI<sub>2</sub>), Rhodamine-6G and Src-Inhibitor-1 were bought from SigmaAldrich, USA. 3,7-Dihydro-1-methyl-3-(2-methylpropyl)-1H-purine-2,6-dione (IBMX), and trans-4-[(1R)-1-Aminoethyl]-N-4pyridinylcyclohexanecarboxamide dihvdrochloride (Y27632) were purchased from Tocris Bioscience. 5-(2,2-Difluoro-benzo[1,3]dioxol-5vlmethylene)-thiazolidine-2.4-dione (AS-604850) and 7-methvl-2-(4morpholinyl)-9-[1-(phenylamino)ethyl]-4H-pyrido[1,2-a]pyrimidin-4-one (TGX221) were purchased from Larodan Fine Chemicals AB, Malmö, Sweden. <sup>35</sup>S]GTP<sub>y</sub>S were bought from Perkin Elmer Inc., USA, Ticagrelor (AZD6140) was a gift from AstraZeneca R&D Mölndal, Mölndal, Sweden.

### Platelet preparation for Light Transmission Aggregation (LTA)

Whole blood was drawn from healthy volunteers through venous puncture (Vacutainer<sup>®</sup> tubes, 0.129 M Na-Citrate, BD, NJ, USA). Whole blood was centrifuged (140 rcf, 20 min, RT) and the supernatant containing platelet rich plasma (PRP) was collected. As a control, platelet poor plasma (PPP) was used and prepared from whole blood through centrifugation (2260 rcf, 10 min,

RT). Each PRP sample contained 250 μl. The LTA measurements were performed using two serially connected Chronolog aggregometers, model 490 (Chronolog, PI, USA). Antagonists were preincubated with PRP for 5 minutes prior to addition to succinate. Aggregation values from LTA experiments are presented as percent of the mean of maximum platelet aggregation (MPA %). Washed platelets were prepared according to the protocol by Cazenave *et al.* [13] before aggregation studies done as described for the PRP.

### Platelet qRT-PCR

(See supplement and reference [7].)

Western blotting on human platelet for SUCNR1. (See supplementary material.)

Confocal immunofluorescence microscopy on platelets (See supplementary material.)

### Calciumflux assay on platelets

Ca<sup>2+</sup>-release, mediated through succinate stimulation, was measured as described earlier by Liu *et al.* [14], with one change in the protocol for the P2Y<sub>1</sub> assay: 0.3 U/ml apyrase was added to the assay buffer to exclude indirect signaling from P2Y<sub>1</sub>. Fluorescence reading was done using a Victor3 1420 Multilabel Counter (Perkin Elmer, USA).

cAMP assay on platelets (See supplementary material.)

Flow cytometric analysis of platelet activation (See supplementary material.)

### Platelet degranulation – ATP release

PRP was prepared as in the LTAmeasurement and was incubated at 37°C in 10 min (1,200 rpm) before addition of the ligand. The released ATP was determined by luminometry in a GloMax<sup>®</sup> TD-20/20 luminometer (Promega, CA, USA), using the ATP SL kit (BioThema, Sweden).

Western blot assay for PKBα /Akt1 determination (See supplement and reference [15].)

### Platelet [<sup>35</sup>S]GTPγS binding

PRP was prepared from ACD-treated whole blood through centrifugation (150 rcf, 20 min, RT) and passed carefully through a Pall Autostop<sup>TM</sup> Leukocyte removal filter (Pall Medical, NY, USA). The filtered PRP was centrifuged (17,000 rcf, 15 min, RT). The pellet was resuspended in a roomtemperature membrane-buffer (20mM HEPES. 20mM EDTA, 150mM NaCl, pH 7.4. RT) and the centrifugation was repeated once. The pellet was resuspended into 3 mL ice-cold membrane-buffer (20mM pН HEPES. 5mM EDTA. 7.4) homogenized for 30 seconds in a Polytron (setting 6), and diluted to 30 ml with ice-cold membrane-buffer (20mM HEPES, 5mM EDTA, pH 7.4). The platelet homogenate was pelleted twice (35,000 rcf, 15 min, 4°C). The final pellet was resuspended in ice-cold membrane buffer (20mM HEPES. 0.6mM EDTA, 5mM MgCl<sub>2</sub>, 0.1mM PMSF, pH 7.4) and stored at -80°C. Protein concentration was measured with a modified Lowry protein assay Kit (Thermo Scientific, USA).

The binding assay was performed essentially as described by Vasiljev et al[16]. Briefly: The final volume of the assay was 200ul and contained 20 ug platelet membranes in binding buffer (20mM HEPES, 100mM NaCl, 5mM MgCl<sub>2</sub>, 10µg/mL saponin, pH 7.4 + 4nM  $[^{35}S]GTP\gamma S.$ 20uM guanosine diphosphate (GDP)). The mixture was incubated (30 min, 30°C) and then filtered through a glass fiber filter (GF/C, Whatman, UK). The filters were washed with 3 x 500µl ice-cold binding buffer and then counted using OptiPhase Hisafe 3 (Perkin Elmer, USA) in a LS 6500 Multi-Purpose Scintillation Counter (Beckman-Coulter, USA).

### Statistical analysis

Calculations and statistics were performed using GraphPad Prism 4.0 software. One-way analysis of variance (ANOVA) was used followed by a Bonferroni correction between groups if there was statistical significance. Student's t - test was used to compare means between two groups. Statistical significance was accepted when P < 0.05. Values are presented as means  $\pm$  SEM, n = number of experiments.

### Ethics

The project was approved by the Ethics Committee of Lund University, Sweden. All participants submitted written consent to take part in the study. The study conforms to US National Institutes of Health guidelines, and the Declaration of Helsinki.

### Results

### Quantification of SUCNR1 in human platelets

The succinate receptor SUCNR1 is platelet expressed in human at approximately the same level as P2Y<sub>1</sub>, (1.28, vs. 1.00, n=6). The expression level of P2Y<sub>12</sub> was about 12-times higher than the expression level of SUCNR1 (n=6) while the expression of A2a was approximately 10-times lower (n=6) (Fig. 1a). The expression of SUCNR1 on human platelets was confirmed with western blot (Fig. 1b) and confocal immunofluorescence microscopy (Fig. 1c). The platelet expression of SUCNR1 was further evaluated using flow cytometry (21.3 % of platelets positive, data not shown) while CHO cells, a cell line previously used to characterize the SUCNR1 receptor, displayed negligible levels of SUCNR1 antibody binding (0.97 % positive, data not shown). CHO cells stained with the SUCNR1 antibody did not display any fluorescence as evaluated with confocal microscopy (see supplementary material).

### Succinate stimulates platelet aggregation

Huang *et al.* [8] has previously demonstrated that succinate potentates agonist-induced platelet aggregation. Using LTA, we tested whether succinate alone is sufficient to activate platelets. Our results clearly demonstrates that succinate independently activates platelets and cause platelet aggregation

in a dose-dependent manner. A typical succinate aggregation displays a biphasic aggregation kinetic. The effect of succinate is half-maximal at 0.3mM and maximal at 0.5mM. To exclude possible disturbances in the serum, we isolated washed platelets and repeated the experiment. This resulted in а displacement of the dose-response curve to the right (Fig. 2a). We could also confirm the potentiating effect of succinate on ADP mediated platelet activation that has been described earlier (Fig. 2b). The effect of succinate on platelet activation was further characterized by demonstrating that both the platelet activation marker for the GPIIb/IIIa. PAC-1. and degranulation marker, P-selectin, were activated (% positive cells) (23.0  $\pm$  3.3 vs.  $6.9 \pm 2.0$ , \*\*=P<0.001, n=16 and 7.6  $\pm$  1.5 vs. 2.7  $\pm$  0.5, \*=P<0.05, n=16) in response to 3mM succinate (Fig. 3a and Fig. 3b). ADP was added as a positive marker both for PAC-1 binding, (58.5  $\pm$ 7.0 vs. 6.9 ± 2.0, \*\*\*=P<0.01, n=16) and for P-selectin,  $(29.1 \pm 5.1 \text{ vs. } 2.7 \pm 2.0,$ \*\*\*=P<0.001, n=16).

### Activation of SUCNR1 inhibits cAMP production in platelets but has no effect on calcium signaling

As the succinate receptor has been reported to be coupled to  $G_{\alpha i}$  activation [9], and is thus expected to result in an attenuation of platelet cAMP production, we tested the effect of 3mM succinate on both forskolin and PGE1 stimulated cAMP production and also on the phosphorylation status of the cAMP/PKA marker VASP. We found that cAMP production in platelets prestimulated by forskolin was significantly reduced for platelets treated with succinate (2.6 $\mu$ M ± 66nM vs. 1.0 $\mu$ M ± 70nM \*\*\*=P<0.001 n=4) This reduction in cAMP also was seen in platelets pre-stimulated with PGE1  $(0.7\mu M \pm 30nM \text{ vs. } 0.4\mu M \pm 7nM,$ \*\*\*=P<0.001, n=4) (Fig. 3c).The decrease in cAMP production was confirmed in VASP phosphorylation (% positive cells)  $(75.4 \pm 6.3 \text{ vs. } 66.7 \pm 7.0.$ \*\*=P=0.0025, n=8) comparing to ADP  $(68.1 \pm 7 \text{ vs. } 23.1 \pm 4.7, ***=P=0.0002,$ n=8) (Fig. 3d).

Succinate (3mM) stimulation of platelets loaded with Fluo-4AM did not result in any change in Ca<sup>2+</sup> mobilization compared to untreated platelets, (5284 RFU ± 6.4 RFU vs. 4963 RFU ± 6.2 RFU, (n.s)=P>0.05, n=4) (Fig. 3e.). Thrombin (1U/mL) elicited a positive calcium response vs. control (7563 RFU ± 185 RFU vs. 4963 RFU ± 6.2 RFU, \*\*\*=P>0.001, n=4) thus demonstrating the functional integrity of the platelets. In order to avoid indirect ADP signaling, mediated through  $P2Y_1$  or caused by ATP release, the experiment was conducted in the presence of apyrase. However, a minor difference could be detected between platelets stimulated with 10µM ADP vs. control (5578 RFU ± 7.7 RFU vs. 4963 RFU ± 6.2 RFU, \*\*\*=P>0.001, n=4).

## Succinate stimulated aggregation is dependent on PI3K $\beta$ activation but not on the G<sub> $\alpha$ 12/13</sub> pathway

As both the PI3K/Akt and the  $G_{\alpha 12/13}$  pathway are involved in mediating platelet aggregation, we used the general phosphoinositide 3-kinase (PI3K) inhibitor wortmannin and the Rho-GEF/ROCK<sup>160</sup>-inhibitor Y27632 to explore the involvement of these

pathways in succinate-stimulated aggregation. While  $10\mu$ M of Y27632 had no effect, there was a significant decrease in succinate-stimulated aggregation after pre-incubating platelets with  $10\mu$ M of wortmannin (75.7 ± 0.8 vs. 1.8 ± 0.7, \*\*\*=P<0.0001, n=6), indicating involvement of the PI3K/Akt pathway (Fig. 4a and Fig. 4b).

In order to identify which PI3K isoform is involved in succinate-induced platelet aggregation we employed TGX221 and AS-604850, which are specific inhibitors for PI3K $\beta$  and PI3K $\gamma$  respectively. 10µM MRS2179 was added in all experiments to avoid potential interference of the P2Y<sub>1</sub> receptor. Stimulating platelets with 3mM succinate in the presence of 1µM AS-604850 resulted in a small, but significant reduction in MPA (72.1  $\pm$  1.1 vs. 77.1  $\pm$  1.5, \*=P<0.05, n=8). However. when AS-604850 was substituting for 1µM of the PI3KB specific TGX221 there was a 99 % reduction in MPA compared to the control (77.1  $\pm$  1.5 vs. 0.6  $\pm$  0.3, \*\*\*=P<0.001, n=8) (Fig. 4c). The involvement of PI3Kβ was confirmed by western blot analysis of Akt phosphorylation. Treatment of platelets with 3mM succinate resulted in Akt phosphorylation that was reversed when 1µM TGX221 was added (Fig. 4d).

The ability of succinate to achieve platelet aggregation in LTA was abolished in the presence of a Src-kinase inhibitor. A statistically significant difference was seen between platelets pre-incubated with  $10\mu$ M Src inhibitor-1 vs. platelets stimulated with 3mM succinate only (74.7 ± 1.4 vs. 7.0 ± 1.2, \*\*\*=P<0.0001, n=7) (Fig. 4e).

## Succinate-stimulated platelet aggregation is dependent on ATP release

Acetyl salicylic acid (ASA) inhibits COX-1 which converts arachidonic acid into thromboxane  $A_2$  $(TXA_2)$ . Pretreatment with 1mM ASA abolished the stimulating effect of 3mM succinate in platelets  $(73.5 \pm 5.2 \text{ vs. } 6.9 \pm 1.2,$ \*=P<0.05, n=14) indicating a role for TXA<sub>2</sub> in succinate-mediated aggregation (Fig. 5a). Since the response of platelets to TXA<sub>2</sub> depends on ADP serving as a positive-feedback mediator, which is required for sustained activation, these results prompted us to investigate the effect of succinate on ATP release. We found that 3mM succinate elicited an approximately 1000-fold increase in ATP release  $(0.3nM \pm 0.1nM \text{ vs. } 0.4\mu\text{M})$ 1.6nM. \*\*\*=P<0.0001, n=6). +confirming the involvement of ATP release in succinate-mediated aggregation (Fig. 6). These results were further strengthened by demonstration that the pro-activating effect induced by 3mM succinate is absent in platelets preincubated with 10µM of the potent  $P2Y_{12}$  antagonist ticagrelor (79.7  $\pm$  0.9 vs. 4.0 ± 0.7, \*=P<0.05, n=12) (Fig. 5b). Platelets pretreated with 10 µM of the P2Y<sub>1</sub> antagonist MRS2179 did on the other hand not display any reduction in MPA  $(76.2 \pm 1.0 \text{ vs. } 74.4 \pm 1.0, \text{ ms. } 74.$ (n.s)P=0.25, n=14), (Fig. 5c).

### Succinate stimulates [<sup>35</sup>S]GTPγS binding to platelet cell membranes

Since demonstration of functional SUCNR1 receptors using standard radioreceptor binding techniques is unpractical due to the low affinity of succinate, we investigated the ability of

[<sup>35</sup>S]GTP<sub>y</sub>S stimulate succinate to binding in platelet plasma membranes. As shown in Fig. 7. increasing concentrations of succinate generated a dose-dependent binding of  $[^{35}S]GTP\gamma S$ with an EC<sub>50</sub>-value of  $100\mu M$  (n=4). Desensitization of the receptor upon ligand stimulation is a well-studied negative regulation mechanism of GPCRs. In order to investigate if the platelet SUCNR1 is subject to homologous or heterologous to desensitization, we prepared plasma membranes from platelets that were prechallenged with either 3mM succinate or 10µM 2MeSADP. The results show that in both cases, the ability of succinate to stimulate  $[^{35}S]GTP\gamma S$  binding was significantly reduced to  $69.6\% \pm 3.4\%$ , (\*\*=P<0.01, n=3) for homologous desensitization and  $81.6\% \pm 2.9\%$ , (\*=P<0.001, n=3) for heterologous desensitization (Fig. 7).

### Discussion

In this study we identified succinate as an independent agonist of full platelet aggregation. Succinate-stimulated platelet aggregation involves activation of the platelet activation marker GPIIb/IIIa and the degranulation marker P-selectin. Succinate stimulation of platelets results in decreased cAMP levels and VASP activation but did not stimulate cytosolic calcium release. Furthermore, succinate-induced platelet aggregation depended on PI3KB/Akt1induced signaling and on Src kinase but did not involve Rho-GEF or PI3Ky. The SUCNR1 is desensitized both homologously by succinate and heterologously by ADP. Finally, our data showed that succinate induces platelet aggregation via TXA2 generation

and ATP/ADP release. Interestingly, we observed a complete abolishment of succinate-mediated platelet aggregation in the presence of the  $P2Y_{12}$  receptor antagonist ticagrelor while the  $P2Y_1$  antagonist MRS2179 had on the other hand no such effect.

The influence of the citric acid cycle metabolite succinate on platelets was first described by Huang et al. [8], who reported that succinate augments the actions of ADP and epinephrine and that this effect was related to attenuation of cAMP levels. In a previous study, using gene array screening, we identified the message of the recently deorphanized succinate receptor SUCNR1 in human platelet mRNA. In an effort to confirm these results we quantified the platelet message of SUCRN1. Interestingly, the level of SUCRN1 expression was the third highest expressed GPCR examined and equaled the levels of P2Y<sub>1</sub> receptor, a receptor with a demonstrated role in platelet activation, thus predicting functionality at the same level.

The succinate receptor is reported to be a low affinity receptor with an EC<sub>50</sub> value for succinate that, depending on the assay method, ranges from 28 µM to 391 uM. These results are well in line with findings with  $EC_{50}$ our an of approximately 300µM based on LTA measurements and at 100 µM for [<sup>35</sup>S]GTPyS binding. Succinate at 3mM activated the marker PAC-1, recognizing and activated GPIIb/IIIa, the degranulation marker P-selectin, thus supporting the picture of an independent platelet activator. Huang et al. [8] reported that succinate treatment resulted in a decrease in platelet cAMP levels. These results correspond well with our findings that show an inhibition of the cAMP/PKA pathway resulting in decreased cAMP levels and, as a consequence, a decrease in PKAmediated phosphorylation of the vasodilator-stimulating phosphoprotein (VASP). Phosphorylated VASP inhibits activation of the membrane bound integrine, GPIIb/IIIa [17] which is an important step for platelet-platelet adhesion, and hence for aggregation of platelets.

In contrast to the report by He et al. [4], we were unable to detect any effect on cytosolic Ca<sup>2+</sup> mobilization. In this study SUCNR1 is described as a receptor capable of coupling not only to  $G_{\alpha i}$ , mediating inhibition of adenylate cyclase, but also to  $G_{\alpha q}$  stimulating  $IP_3$  turnover and  $Ca^{2+}$  release. However, only a part of the IP<sub>3</sub> signal could be attributed to  $G_{\alpha q}$  activation as the other half was sensitive to pertussis toxin and thus likely caused by  $G_{\beta\gamma}$  mediated activation of PLCB [18]. Furthermore, in a recent report by Hakak et al. [9] where endogenous succinate receptors were studied in TF-1 cells, the pertussis toxin insensitive part of the IP<sub>3</sub> signal was absent. Those authors suggest that since the results by He et al. [4] were obtained in recombinant CHO cells, overexpressing SUCNR1, it is likely that the PTX-insensitive signal is an artifact, caused by promiscuous G-protein coupling. Our results support the view of SUCNR1 as a receptor primarily coupled to  $G_{\alpha i}$  and we believe that the absence of a succinate stimulated Ca<sup>2+</sup> signal in platelets can be explained by that the lack of G<sub>by</sub>-mediated activation of PLCB in platelets. There are reports that support the absence of  $G_{\beta\gamma}$ -mediated Ca<sup>2+</sup> signaling in platelets: for example, it has been demonstrated that while stimulation of the P2Y<sub>12</sub> receptor results

in  $G_{\alpha i}$ -dependent activation of PI3K, it will not result in  $Ca^{2+}$  mobilization [19]. While it is currently not known which  $G_{\beta\gamma}$  combinations are expressed in platelets it has been shown that the type of the  $G_{\beta}$  subunit determines whether PI3K or PLC $\beta$  activation is favored [20], [21].

Although we could detect no effect on calcium mobilization, we obtained a dose dependent increase in [<sup>35</sup>S]GTPyS binding upon stimulation with succinate, thus confirming the involvement of  $G_{\alpha i}$ . Furthermore, the importance of  $G_{\beta\gamma}$ mediated signaling was confirmed by a strong PI3 kinase activation resulting in Akt/PKB phosphorylation. The responsible isoform turned out to be PI3K $\beta$ , which is somewhat surprising, as PI3K $\gamma$  is the isoform that usually is associated with stimulation by  $G_{\beta\gamma}$ . However, PI3KB has previously been identified as the dominant PI3K isoform responsible for G<sub>i</sub> mediated GPIIb/IIIa activation following ADP stimulation of the P2Y<sub>12</sub> receptor [22]. The Src kinases have recently been reported by Nash et al. [23] to be involved in adrenergic Gicoupled signaling in platelets. Nash et al. described that Src kinases acting downstream of the  $\alpha_{2a}$  receptor mediate both the primary and secondary wave of aggregation. They also demonstrated that P2Y<sub>12</sub> mediated aggregation is dependant on Src kinases and suggested this pathway to be common for Gicoupled receptors. This finding led us to investigate if Src kinases could be involved in SUCNR1 activation as well. Indeed, we found that

platelet aggregation, induced by succinate, was nearly abolished, from approximately 75 to 7 % MPA, in the presence of a Src kinase inhibitor.

The importance of a positive feedback was demonstrated by the finding that succinate stimulated а massive ATP/ADP release and was confirmed both by the sensitivity to the  $P2Y_{12}$ antagonist ticagrelor and by the increase of the vesicular-inner membrane bound protein P-selectin. Negative regulation of G-protein coupled receptors is triggered phosphorylation leading bv to desensitisation and internalization of the receptor. Our results demonstrate that the platelet SUCNR1 is rapidly desensitized challenge upon with succinate. Previously Robben et al. [24] showed that SUCNR1 is similarly desensitised in Madin-Darby Canine Kidney cells.

Interestingly, the platelet SUCNR1 also seems to be subject to heterologous desensitization through activation of either P2Y<sub>12</sub> and/or P2Y<sub>1</sub>. Hardy et al [25] have previously reported that the desensitization mechanism triggered by  $P2Y_1$  and  $P2Y_{12}$  differ. While  $P2Y_{12}$  is desensitized through a homologous mechanism involving GRK phosphorylation and receptor internalization, P2Y1 desensitization is to a great extent dependant on PKC which phosphorylate ligand-bound and inactive GPCRs in a heterologous manner [26]. Thus it seems likely that the observed cross-desensitization of SUCNR1 by 2MeSADP is caused by stimulation of  $P2Y_1$  rather than  $P2Y_{12}$ .

When discussing the physiological implications of succinate-mediated platelet activation it is important to note that the succinate levels in serum generally are too low to permit activation of SUCNR1 [27]. However, under pathological conditions such as animal models of type 2 diabetes and during different forms of ischemia, succinate concentrations have been reported to rise to well up to millimolar ranges *[*10. 11]. The potential importance of succinate/SUCNR1 in cardiovascular disease was highlighted in a recent publication showing that succinate, at concentrations equal to ischemic levels, modulate apoptosis in rat ventricular cardiomvocvtes Aguiar et al. [28] and that this effect is mediated by SUCNR1. Moreover, succinate has been suggested as a myocardial marker of ischemia/reperfusion injuries by Kakinuma et al. [29]. Thus, succinate could be important during myocardial infarction, both via apoptotic effects on the cardiomyocyte and by causing platelet aggregation and thereby epicardial vessel occlusion or microembolism. Since succinate is ubiquitous in all cell types, the physiological role could potentially be very important. However, to prove this hypothesis. selective succinate antagonists are needed to perform in vivo experiments or even clinical studies.

As for many platelet activators, succinate activation is dependent on the positive feedback system thromboxane and ADP for full platelet aggregation. Succinate-induced platelet aggregation by inhibited aspirin and was interestingly, also by the potent  $P2Y_{12}$ receptor antagonist ticagrelor. Ticagrelor was recently tested in the phase III study PLATO, where it was superior to clopidogrel and reduced both myocardial infarctions and mortality [30]. It is possible that some of the beneficial effects of aspirin and ticagrelor are due to an inhibition of succinate-induced platelet aggregation.

In conclusion, the succinate receptor is one of the most expressed receptors on human platelets. It can stimulate platelet aggregation by itself and increase Pselectin and GPIIb/IIIa expression. The effects are mediated via the cAMP-PKA-VASP pathway, but also depend on Src kinase activation and PI3K $\beta$ /Akt1induced signaling. Succinate-induced platelet activation can be prevented with aspirin or ticagrelor, and this observation may explain some of their beneficial effects. It is possible that selective succinate receptor blockers could have further effects in preventing thrombosis and could have a place in the treatment of cardiovascular disease.

### Addendum

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

### Acknowledgements

The study has been supported by the Swedish Heart and Lung Foundation, Swedish Scientific Research Council, ALF and Lund University Hospital funds.

### **Disclosure of Conflict of Interests**

The authors state that they have no conflict of interest.

### References

- Sapieha, P., et al., *The succinate receptor GPR91 in neurons has a major role in retinal angiogenesis.* Nat Med, 2008. 14(10): p. 1067-76.
- Gonzalez, N.S., et al., The fate of P2Y-related orphan receptors: GPR80/99 and GPR91 are receptors of dicarboxylic acids. Purinergic Signal, 2004. 1(1): p. 17-20.
- Wittenberger, T., H.C. Schaller, and S. Hellebrand, An expressed sequence tag (EST) data mining strategy succeeding in the discovery of new G-protein coupled receptors. J Mol Biol, 2001. 307(3): p. 799-813.
- He, W., et al., Citric acid cycle intermediates as ligands for orphan G-protein-coupled receptors. Nature, 2004. 429(6988): p. 188-93.
- Toma, I., et al., Succinate receptor GPR91 provides a direct link between high glucose levels and renin release in murine and rabbit kidney. J Clin Invest, 2008. 118(7): p. 2526-34.
- Macaulay, I.C., et al., Comparative gene expression profiling of in vitro differentiated megakaryocytes and erythroblasts identifies novel activatory and inhibitory platelet membrane proteins. Blood, 2007. 109(8): p. 3260-9.
- Amisten, S., et al., Gene expression profiling for the identification of G-protein coupled receptors in human platelets. Thromb Res, 2008. 122(1): p. 47-57.

- Huang, E.M., E.B. McGowan, and T.C. Detwiler, *Succinate potentiates the action of platelet agonists.* Thromb Res, 1984. 36(1): p. 1-8.
- Hakak, Y., et al., The role of the GPR91 ligand succinate in hematopoiesis. J Leukoc Biol, 2009. 85(5): p. 837-43.
- Sadagopan, N., et al., Circulating succinate is elevated in rodent models of hypertension and metabolic disease. Am J Hypertens, 2007. 20(11): p. 1209-15.
- Correa, P.R., et al., Succinate is a paracrine signal for liver damage. J Hepatol, 2007. 47(2): p. 262-9.
- Wust, J., Presumptive diagnosis of anaerobic bacteremia by gasliquid chromatography of blood cultures. J Clin Microbiol, 1977. 6(6): p. 586-90.
- 13. Cazenave, J.P., et al., *Preparation of washed platelet suspensions from human and rodent blood.* Methods Mol Biol, 2004. **272**: p. 13-28.
- Liu, E.C. and L.M. Abell, Development and validation of a platelet calcium flux assay using a fluorescent imaging plate reader. Anal Biochem, 2006. 357(2): p. 216-24.
- Tan, C., et al., ADP receptor P2Y(13) induce apoptosis in pancreatic beta-cells. Cell Mol Life Sci. 67(3): p. 445-53.
- 16. Vasiljev, K.S., A. Uri, and J.T. Laitinen, 2-Alkylthio-substituted platelet P2Y12 receptor antagonists reveal pharmacological identity between the rat brain Gi-linked ADP receptors and P2Y12.

Neuropharmacology, 2003. **45**(1): p. 145-54.

- 17. К., Horstrup. et al.. **Phosphorylation** of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. Eur J Biochem, 1994. 225(1): p. 21-7.
- Ford, C.E., et al., Molecular basis for interactions of G protein betagamma subunits with effectors. Science, 1998. 280(5367): p. 1271-4.
- Mathieu, R., et al., Synthesis and biological activity of 2-alkylated deoxyadenosine bisphosphate derivatives as P2Y(1) receptor antagonists. Bioorg Med Chem, 2004. 12(7): p. 1769-79.
- Maier, U., A. Babich, and B. Nurnberg, Roles of non-catalytic subunits in gbetagamma-induced activation of class I phosphoinositide 3-kinase isoforms beta and gamma. J Biol Chem, 1999. 274(41): p. 29311-7.
- 21. Dupre, D.J., et al., The role of Gbetagamma subunits in the organization, assembly, and function of GPCR signaling complexes. Annu Rev Pharmacol Toxicol, 2009. **49**: p. 31-56.
- 22. Kim, S., et al., Role of phosphoinositide 3-kinase beta in glycoprotein VI-mediated Akt activation in platelets. J Biol Chem, 2009. **284**(49): p. 33763-72.
- 23. Nash, C.A., et al., *Src kinases are* essential for primary aggregation by *G*-coupled receptors. J Thromb Haemost.

- Robben, J.H., et al., Localization of the succinate receptor in the distal nephron and its signaling in polarized MDCK cells. Kidney Int, 2009. 76(12): p. 1258-67.
- 25. Hardy, A.R., et al., *P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinasedependent mechanisms.* Blood, 2005. **105**(9): p. 3552-60.
- Ferguson, S.S., Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev, 2001.
  53(1): p. 1-24.
- 27. Kushnir, M.M., et al., Analysis of dicarboxylic acids by tandem mass spectrometry. Highthroughput quantitative

*measurement of methylmalonic acid in serum, plasma, and urine.* Clin Chem, 2001. **47**(11): p. 1993-2002.

- Aguiar, C.J., et al., Succinate modulates Ca(2+) transient and cardiomyocyte viability through PKA-dependent pathway. Cell Calcium, 2009.
- Kakinuma, Y., et al., Myocardial metabolic markers of total ischemia in vitro. Nagoya J Med Sci, 1994. 57(1-4): p. 35-42.
- Wallentin, L., et al., *Ticagrelor versus clopidogrel in patients with acute coronary syndromes.* N Engl J Med, 2009. 361(11): p. 1045-57.

### **Supplementary material**

### Platelet qRT-PCR

Platelet extraction for RNA isolation. cDNA synthesis and aRT-PCR. followed the methodology described by Amisten *et al.* [7]. cDNA was synthesized using Fermentas RevertAid H MinUS First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturers instructions. gReal-Time PCR was performed in a LightCycler<sup>®</sup> (Roche, Switzerland) using Maxima TM SYBR Green qPCR (x2) mix (Fermentas). Primer sequences used for RT-PCR are listed in table 1. The data was normalized to  $\beta$ -actin and P2Y<sub>1</sub>, essentially as previously described by Amisten et al. [7].

### Western blotting on human platelet for SUCNR1.

Protein was extracted from isolated human platelets and chinese hamster ovary (CHO) cells with SDS sample buffer (62.5 mmol/l Tris-HCl, 2% SDS, and 10% glycerol). Caki-1 cells in loading buffer were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Equal amounts of protein were separated by SDS-PAGE on 12.5% Tris-HCl gels and transfered to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked in 5% non-fat dry milk and subsequently incubated with primary antibody against SUCNR1 (goat polyclonal; 1:1000 dilution in 5% milk),

and secondary horse peroxidaseconjugated antibody (1:10,000 dilution in 5% milk). Both antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bands were detected using chemiluminescence (Supersignal West Femto, Pierce Biotechnology, Rockford, IL, USA).

### Confocal immunofluorescence microscopy on platelets

Platelets were isolated as previously described by Liu et al. [14] and fixed in 0.75% parformaldehyde for 15 minutes and subsequently washed in phosphate buffered saline (PBS). After 2 hours blocking in room temperature with 2% bovine serum albumin (BSA), platelets were incubated over night at 4°C with primary goat polyclonal anti-SUCNR1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:25. Several washing steps were performed and platelets were incubated for 2 hours at room temperature with a secondary donkey anti-goat Alexa 488 antibody diluted 1:500. Finally platelets were incubated with rhodamine-6G (0.02 g/dl) for 20 minutes. The platelets were let to adhere to coverslips over night in a humid chamber and were subsequently mounted with Polyscience Mounting Media (Aqua Poly Mount, Polyscience Inc.). Control of unspecific staining was performed by omitting the primary or secondary antibody from the protocol. Confocal images were acquired with a Zeiss LSM 510 laser scanning confocal microscope using a 100X objective.

### Confocal immunofluorescence microscopy on CHO cells

CHO cells cultured on coverslips were fixed in 4% formaldehyde in PBS and blocked with 2% BSA in PBS. Primary antibody, goat anti-GPR91 (1:25, Santa Cruz Biotechnology) and secondary antibody, Cv5-anti-goat IgG (1:1000. Abcam) were used at excitationemission wavelengths of 633 and >650 nm, respectively. The fluorescent nucleic acid dve SYTOX Green (1:3.000. Molecular Probes) was used for nuclear identification. Cells were examined at x40 in a Zeiss LSM 5 Pascal laser scanning confocal microscope. Result from CHO study is presented in Fig 1 supplementary material.

### cAMP assay on platelets

PRP prepared as described earlier was incubated in the presence of 1mM acetylic salicylic acid (ASA), 0.3U/mL apyrase (15 min, RT). After incubation, the PRP was further incubated with 5mM EDTA and centrifuged (700 rcf. 10 min RT). The supernatant was discarded and the PRP-pellet was resuspended in HEPES-Tyrode buffer (145mM NaCl, 5mM KCl, 0.5mM Na<sub>2</sub>HPO<sub>4</sub>, 1mM MgSO<sub>4</sub>, 10mM HEPES, 5mM glucose, 0.3U/mL apyrase, pH 7.4), and pelleted again. The supernatant was removed and the pellet was resuspended to give 1.65 x 10<sup>8</sup> cells/mL in modified HEPES-Tyrode buffer (145mM NaCl. 3mM KCl. 0.5mM Na<sub>2</sub>HPO<sub>4</sub>, 1mM MgSO<sub>4</sub>, 10mM HEPES, 5mM glucose, 0.6U/mL apyrase, 1mM IBMX - phosphatase inhibitor, pH 7.4). 0.2 x10<sup>8</sup> platelets were incubated for 15 min at 37°C before addition of stimulants. After 10 minutes platelets were pelleted by centrifugation at 800rcf for 5min. The platelets were lysed in 100µL of 0.1M HCl and pelleted again (1200 rpm, 5 min, 4°C). The supernatant was stored in -80°C until analysis. For measuring the stimulated platelets a Cyclic AMP EIA assay (Cavman Chemical, MI, USA) was used following the manufacturers instructions. The readout of the results from the cAMP assay was performed in а Victor3 spectrophotometer, 1420 Multilabel Counter. (Perkin Elmer. USA).

### Flow cytometric analysis of platelet activation

Whole blood was collected into 0.129M Na-Citrate, (Vacutainer®, BD, CA, USA). Succinate stimulation (3mM) of platelet vasodilator-stimulated phosphoprotein (VASP) was measured PLT VASP/P2Y12-kit using the (Biocytex, Marseille, France). For Pselectin and GPIIb/IIIa activity, whole blood was incubated in HEPES-saline buffer (145mM NaCl, 5 mM KCl, 10 mMHEPES 7.4) рH with labelled fluorescently monoclonal antibodies against P-selectin (CD62P) and GPIIb/IIIa (PAC-1) or corresponding isotype control antibodies (BD, CA, USA). To induce platelet activation, 3 mM of succinate and 0.1 µM ADP was added. After 15 minutes in the dark at room temperature 0.2 % parformaldehyde was added to fix the cells. All samples were diluted 1:20 in PBS and analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, CA, USA). The platelet population was gated based on forward and side scattered light. A threshold for nonspecific fluorescence was set using isotype control antibodies and all events

above that value were considered positive. A total of 3000 events were collected in the platelet gate from each sample.

### Western blot assay for PKBa /Akt1 determination

Whole blood was collected in ACD-A Vacutainer<sup>®</sup> tubes as described earlier. acidified whole blood The was centrifuged to extract the PRP (230 rcf. 20 min, RT). The PRP fraction in the supernatant was aspired into a fresh tube. The PRP was incubated (45 min, 37°C) with 1mM ASA. After the incubation the PRP was centrifuged (980 rcf, 10 min, RT) and the platelet rich pellet was collected and resuspended in modified Tyrodes-HEPES buffer; (138mM NaCl, 2.7mM KCl, 2mM MgCl<sub>2</sub>, 0.42mM NaH<sub>2</sub>PO<sub>4</sub>, 5mM glucose. 10mM HEPES, 0.2% BSA, 0.1U/ml apyrase. pH 7.4). Cell count was set to  $2*10^8$ cells per mL. Cell suspension was aliquot in concentrations of 1\*10<sup>8</sup> cells per sample, and pre-incubated in 37°C before addition of specific inhibitors and further stimuli. Following western blot procedure and analysis were performed as previously described by Tan et al. [15]

### Figure 1. (A, B and C).



**Fig. 1.** (A) Upper left: Quantitative Real-Time PCR analysis of the SUCNR1,  $P2Y_{12}$  and  $A_{2a}$  expression levels in platelets.  $P2Y_1$  was used as reference gene. (B) Upper right: Expression of SUCNR1 protein in platelets (Plt). SUCNR1 protein (38 kDa) was detected in human platelets by Western blot. Caki-1 and CHO cells were used as positive and negative controls, respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as loading control. Data are representative for two experiments. (C) Lower centre: Representative confocal image of SUCNR1 protein expression (green) in human washed platelets stained with rhodamine-6G (red). Right panel; merged images. Bar 10 $\mu$ m.



**Fig. 2.** (A) Upper left: Dose-response curve presenting platelet PRP aggregation mediated through elevated doses of succinate. Time for aggregation was 6 minutes; data were collected from six experiments. Upper right: Curve diagram with representative aggregation curves from each experiment. Lower left: Dose-response curve presenting aggregation of washed platelets mediated by elevated doses of succinate. Time for aggregation was delayed through the washing procedure, to 16 minutes: data were collected from six experiments. Lower right: Curve diagram illustrates representative aggregation curves from each experiment. (B) Lower bar graph: Potentiating effect of succinate on ADP-mediated platelet activation in washed platelets (ANOVA: \*\*=P<0.05, \*\*\*=P<0.001, n=5).





**Fig. 3.** (A) Results from a flow cytometry study on GPIIb/IIIa activation in platelets. Upper left: The PAC-1 antibody was used to detect platelet with activated GPIIb/IIIa. There was marked differences between un-stimulated platelets and platelets stimulated with 3mM succinate or 5 $\mu$ M ADP (ANOVA: \*\*=P<0.01, n=16). (B) Upper right: Flow cytometry study on P-Selectin release from platelets. Vesicle secretion of  $\alpha$ -granules mediated by succinate could be statistically confirmed between untreated platelets vs. platelets treated with 3mM succinate or 5 $\mu$ M ADP (ANOVA: \*=P<0.05, \*\*\*=P<0.001, n=16). (C) Lower centre: Effects of succinate on forskoline and PGE1 induced cAMP-production in platelets. 1\*10<sup>8</sup> washed platelets were used per sample. Significant differences in cAMP production is observed between: platelets pretreated with 3mM succinate and 10 $\mu$ M forskolin vs. 10 $\mu$ M forskolin (ANOVA: \*\*\*=P<0.001, n=4) and platelets pretreated with 3mM succinate and 10 $\mu$ M succinate and 100nM PGE1 (ANOVA: \*=P<0.05, n=4).

### Figure 3 (D and E).



**Fig 3. (D)** Upper panel: Flow cytometry study on platelets measuring the ratio of phosphorylated/dephosphorylated vasodilator-stimulated phosphoprotein (VASP). Left bar graph represents the dephosphorylating effect of 3mM succinate on VASP on PGE1 stimulated platelets (Student's *t*-test: \*\*=P=0.0025, n=8). Right bar graph represent the dephosphorylating effect of ADP on VASP on PGE1 stimulated platelets (Student's *t*-test: \*\*\*=P=0.0002, n=8). (E) Lower panel: Calcium signaling in platelets. Left bar graph: Data are presented as relative fluorescence units (RFU). No significant difference between platelets treated with succinate vs. control (ANOVA: (n.s)=P>0.05, n=4). Significance were seen between platelets stimulated with thrombin vs. control (ANOVA: \*\*\*=P<0.001, n=4). Difference were also seen in ADP vs. control (ANOVA: \*\*\*=P<0.001, n=4). Right graph illustrating peak values from experiments: (**n**) vehicle (control), ( $\circ$ ) 3mM succinate, (•) 10 $\mu$ M ADP and ( $\Box$ ) 1U/mI thrombin.



**Fig. 4.** (A) Upper panel: PRP pretreated with 10μM of the Rho-GEF/ROCK<sup>160</sup>-inhibitor, Y27632 (blocking  $G_{12/13}$  –pathways) and stimulated with 3mM succinate vs. PRP stimulated with 3mM succinate only (Student's *t* - test: (n.s)=P=0.6, n=4). (B) Centre panel: PRP pretreated with 10μM wortmannin and stimulated with 3mM succinate vs PRP stimulated with 3mM succinate only, (Student's *t*-test: \*\*\*=P<0.001, n=6). The LTA-curves accompanying the bar charts represent the characteristic aggregation pattern from each of the experiments. (C) Lower panel: LTA on PRP pre-incubated with AS-604850 (PI3Kγ-inhibitor) and TGX221 (PI3Kβ-inhibitor). Samples were further stimulated with 3mM succinate. Platelets pre-incubated with 1μM AS-604850 showed a moderate significance (ANOVA: \*=P<0.05, n=8). However platelets pre-incubated with 1μM TGX221 displayed an augmenting statistical difference (ANOVA: \*\*\*=P<0.001, n=8).

### Figure 4 (D and E).



**Fig 4. (D)** Upper panel: Western blot (including bar chart) of PKB $\alpha$ /Akt 1 on washed platelets treated with 1 $\mu$ M of the PI3K $\gamma$  inhibitor AS-604850, or 1 $\mu$ M of the PI3K $\beta$  inhibitor TGX221. All platelet samples were stimulated with 3mM succinate, except for un-stimulated platelets which represent the negative control. (ANOVA: \*\*\*=P<0.001, \*\*=P<0.01, n=3). (E) Lower panel: Inhibition of Src-kinase by 10 $\mu$ M Src Inhibitor-1 on 3mM succinate stimulated PRP (Student's *t*-test: \*\*\*=P<0.0001, n=7).



**Fig. 5.** (A) Upper panel: Succinat stimulated PRP vs. PRP pre-treated with 1mM ASA and further stimulated with succinate. There is a statistical significance between succinate stimulated platelet aggregation in the presence and absence of ASA (Student's *t*-test: \*\*\*=P<0.0001, n=14). (B) Centre panel:  $P2Y_{12}$  dependence in succinate stimulated platelet aggregation. Significant difference was found between PRP stimulated with 3mM succinate vs. PRP pre-treated with 10µM of the  $P2Y_{12}$  inhibitor ticagrelor and further stimulated with succinate (Student's *t*-test: \*\*\*=P<0.0001, n=13). (C) Lower panel:  $P2Y_1$  dependence of succinate-stimulated platelet aggregation. No statistical difference was seen between untreated PRP vs PRP pre-treated with the  $P2Y_1$  antagonist MRS2179 (Student's *t*-test: (n.s)=P=0.25. n=16). The LTA-curves accompanying the bar charts represent the characteristic aggregation pattern from each of the experiments.

### Figure 6.



**Fig. 6.** Succinate mediated ATP release. Succinate is capable of stimulating ATP release in PRP. (Student's *t*-test: \*\*\*=P<0.0001, n=6).

Figure 7.



**Fig. 7.** Dose-response of succinate-stimulated [ $^{35}$ S]GTP $\gamma$ S-binding in platelet membranes. EC<sub>50</sub>-value equals 110 $\mu$ M. untreated platelet membranes (**n**), platelet membranes pre-treated with 10 $\mu$ M 2MeSADP ( $\circ$ ), platelet membranes pre-treated with 3mM succinate ( $\bullet$ ). The maximal response, obtained for membranes pretreated with either 10 $\mu$ M 2MeSADP or 3mM succinate, was significantly reduced compared to untreated membranes (ANOVA: \*\*=P<0.001, \*=P<0.01, n=3).

### Supplementary material: Högberg et al.

Gene name	Forward primers	Reverse primers	Annealing
			temp. °C
ACTB	5'-CTACCTCATGAAGATCCTCACCGA -3'	5'-CGCTCATTGCCAATGGTGAT -3'	60
P2Y1	5'-ATCTGTATCAGCGTGCTGGTGTG-3'	5'-GCCCAGAATCAGCACCAAGG-3'	60
P2Y12	5'-CTATCGATCGCTACCAGAAGAC-3'	5'-TCATGCCAGACTAGACCGAA-3'	60
SUCNR1	5'-TGTGTCTAACACTGTTGGGGGTTCC-3'	5'-TCCTCACATTCCGCATGACG-3'	60
ADORA2A	5'-TGCTGGCTGCCCCTACACAT-3'	5'-TAAGCGTCGGTGCAGGACTC-3'	62

Table 1. Primers used for RT-PCR assay (Amisten <i>et al.</i> [7]	Table 1	. Primers used	for RT-PCR	assay (Amisten	et al. [7])
---	---------	----------------	------------	----------------	-------------



**Fig. 1** Representative image of CHO cells stained for SUCNR1 (green). Upper left: SUCNR1 staining. Lower left: Nuclear staining (red). Right: Merged. Scale bar 50 µm.

# Paper IV

# Farnesyl pyrophosphate, an endogenous antagonist to ADP stimulated P2Y<sub>12</sub> receptor mediated platelet aggregation

C. Högberg\*, O. Gidlöf\*, F. Deflorian†, K.A. Jacobson†, B. Olde\*, D. Erlinge\*

\*Department of Cardiology, Lund University Hospital, SE-221 84 Lund, Sweden †National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD 20892, USA

(Manuscript)

### Abstract

*Background:* Farnesyl pyrophosphate (FPP) is an intermediate in the mevalonate synthetic pathway, but it has also been reported to activate LPA platelet receptors. The aim of this study was to investigate the role of FPP in platelet aggregation.

*Methods and Results:* Human platelets were studied with light transmission aggregometry, flow cytometry and [ $^{35}$ S]GTP $\gamma$ S assays. As shown previously, FPP could potentiate LPA stimulated shape change. Surprisingly, FPP also acted as a selective non-competitive antagonist to ADP induced platelet aggregation. FPP blocked ADP-induced cAMP inhibition and [ $^{35}$ S]GTP $\gamma$ S binding in platelets. In CHO cells expressing P2Y<sub>12</sub> receptors FPP caused a rightward shift of the [ $^{35}$ S]GTP $\gamma$ S binding curve. Docking of FPP in a P2Y<sub>12</sub> receptor model revealed molecular similarities with ADP and a good fit into the binding pocket for ADP. *Conclusions:* FPP is a noncompetitive antagonist of ADP induced P2Y<sub>12</sub> mediated platelet aggregation. It could be an endogenous antithrombotic factor modulating the strong platelet aggregating effects of ADP in a similar way as we use clopidogrel, prasugrel or ticagrelor in the treatment of ischemic heart disease.

Short title: Farnesyl pyrophosphate is an antagonist of P2Y<sub>12</sub> receptors.

### Corresponding author

David Erlinge, MD, PhD, Professor Dept. of Cardiology, Lund University Skåne University Hospital S-221 85 Lund, Sweden Phone: +46 46 17 25 97 Fax: +46 46 15 78 57 david.erlinge@med.lu.se
#### Introduction

Farnesyl pyrophosphate (FPP) is an intermediate in the mevalonate synthetic pathway, an important metabolic route controlling the cholesterol production, which is present in all eukarvotic animals. Goldstein et al. [1]. FPP is formed by sequential condensation of dimethylallyl pyrophosphate (DMPP) and 3isopenthenyl pyrophosphate (IPP). Besides the function as a precursor in the cholesterol synthesis FPP serves as a donator in post-translational isoprenylation of proteins both directly, and indirectly by being a intermediate for geranylgeranyl pyrophosphate (GGPP) McTaggart et al. [2].

Although FPP is synthesized in the cytoplasm, it can be detected in human plasma. The steady-state plasma level was reported to be 6.6 ng/ml (17 nM), but even the mild physiological alteration caused by eating a meal have been demonstrated to increase the plasma concentration 200 times. Very little is known about plasma levels of FPP during e.g. ischemia. Since HMG-CoA reductase is the main target for statins, the major group of cholesterol lowering medications, it is likely that this kind of therapy also affects the plasma FPP levels.

While the basic function of FPP seems to be structural, there have recently been several reports indicating a role also in signalling. FPP was shown to act as a natural antagonist on the LPA<sub>2</sub> and LPA<sub>3</sub> receptors while it was reported to be an agonist at the structurally divergent LPA<sub>4</sub> and LPA<sub>5</sub> receptors, Williams et al. [3]. In an earlier report we inventoried the different G-protein coupled receptors present in platelet cDNA and found high expression levels of LPA<sub>5</sub> and LPA<sub>4</sub> mRNA. As there have been conflicting reports regarding the function of FPP, on platelets, we decided to investigate the function. Somewhat surprisingly we discovered that the main function of FPP is

not as an agonist at the LPA<sub>4</sub> and LPA<sub>5</sub> receptors but rather as an antagonist at the structurally related and in the platelet activation process most important adenosine di-phosphate (ADP) receptor  $P2Y_{12}$ . Furthermore, the molecular structure of FPP is similar to ADP in having a di-phosphate side chain.

Here we demonstrate that FPP attenuates platelet aggregation by competing with 2-MeSADP, both at the receptor site and on the functional level.

#### Ethics

The Ethics Committee of Lund University approved the project. All volunteers submitted written consent to participate in the study. The study conforms to US National Institutes of Health guidelines, and the Declaration of Helsinki.

#### **Calculations and statistics**

Calculations and statistics were performed using GraphPad Prism 4.0 software. Statistical significance was accepted when P< 0.05, using One-way ANOVA or Student's *t* - test analysing the data. Values are presented as means  $\pm$  SEM. n = number of tests.

#### Chemicals

3,7,11-Trimethyl-2,6,10-dodecatrien-1-yl pyrophosphate ammonium salt (FPP), 3,7,11-Trimethyl-2,6,10-dodecatrien-1-yl monophosphate ammonium salt (FMP), all trans-3,7,11,15-Tetramethyl-2,6,10,14hexadecatetraenvl pyrophosphate (GGPP), trans-3,7ammonium salt pyrophosphate Dimethyl-2,6-octadienyl ammonium (GPP), Isopentenyl salt

pyrophosphate triammonium salt (IPP),  $\gamma,\gamma$ -Dimethylallyl pyrophosphate triammonium salt (DMAPP), 2-Methylthio adenosine-5'-diphosphate (2-MeSADP), Thrombin Receptor Activator Peptide (TRAP) and Epinephrine was bought from SigmaAldrich, USA. [<sup>35</sup>S]GTP $\gamma$ S was bought from Perkin Elmer Inc., USA. Ticagrelor (AZD6140) was a gift from, AstraZeneca R&D Mölndal, Mölndal, Sweden. Fluo4-AM was from Invitrogen,

#### Methods and material

#### Platelet preparation for Light Transmission Aggregation (LTA)

Aggregation studies on human platelet poor plasma (PRP) were done as previously described by Högberg *et al.*[4]. Preparation for aggregation studies on washed platelets were performed as described by Cazenave *et al.* [5].

## Flow cytometric analysis of platelet activation

Whole blood was collected into 0.129M Na-Citrate, (Vacutainer®, BD, CA, USA). For P-selectine and GPIIb/IIIa activity, whole blood was incubated in HEPESsaline buffer (145mM NaCl, 5mM KCl, 10 mM HEPES pH 7.4) with fluorescently labelled monoclonal antibodies against Pselectin (CD62P) and GPIIb/IIIa (PAC-1) or corresponding isotype control antibodies (BD, CA, USA). To induce platelet activation, 30 µM of FPP and 0.1µM ADP was added. After 15 minutes in the dark at room temperature 0.2 % parformaldehyde was added to fix the cells. All samples were diluted 1:20 in PBS and analyzed on a Coulter EPICS XL flow cytometer (Beckman Coulter, CA, USA). The platelet population was gated based on forward and

side scattered light. A threshold for nonspecific fluorescence was set using the isotype control antibodies and all events above that value were considered positive. A total of 3.000 events were collected in the platelet gate from each sample.

#### Calciumflux assay on platelets

PRP for studies on internal  $Ca^{2+}$ -release mediated through succinate stimulation, was prepared as earlier described by Liu *et al* 2006 [6], with the following modifications: 5mM Fluo4-AM was used instead of 2mM. Fluorescence reading was done using a Victor3 spectrophotometer, 1420 Multilabel Counter, (Perking Elmer, USA).

### Platelet [<sup>35</sup>S]GTPγS binding

PRP was prepared from ACD treated whole blood through centrifugation (150 rcf. 20 min. RT) and passed carefully through a Pall Autostop<sup>TM</sup> Leukocyte removal filter, (Pall Medical, NY, USA). The filtrated PRP was centrifuged (17,000 rcf, 15 min, RT). The pellet was resuspended in а room tempered membrane-buffer (20mM HEPES, 20mM EDTA, 150mM NaCl, pH 7.4, RT) and the centrifugation was repeated once. The pellet was first resuspended into 3mL icecold membrane-buffer (20mM HEPES, 5mM EDTA, pH 7.4) then homogenized for 30 seconds in a Polytron (setting 6), and deluted to 30ml with ice-cold membrane-buffer (20mM HEPES, 5mM EDTA, pH 7.4). The platelet homogenate was pelleted twice (35,000 rcf, 15 min, 4°C). The final pellet was resuspended in ice-cold membrane buffer (20mM HEPES, 0.6mM EDTA, 5mM MgCl<sub>2</sub>, 0.1mM PMSF, pH 7.4) and stored at -80°C. Protein concentration was measured with a

modified Lowry protein assay Kit (Thermo Scientific, USA).

The binding assav was performed essentially as described by Vasiljev et al. [7]. Breifly: The final volyme of the assay was 200µl and contained 20µg platelet membranes in binding buffer (20mM HEPES, 100mM NaCl, 5mM MgCl<sub>2</sub>,  $10\mu g/mL$  saponin, pH 7.4 + 4nM  $[^{35}S]GTP\gamma S$ , 20µM guanosine diphosphate (GDP)). The mixture was incubated (30 min, 30°C) and then filtrated through glass fiber filter (GF/C, Whatman, UK). The filters were washed with 3 x 500µl ice cold binding buffer and then counted using OptiPhase Hisafe 3 (PerkingElmer Inc. USA) in a Liquid Scintillation Counter (Beckman Coulter Inc. USA).

#### $[^{35}S]GTP\gamma S$ binding on P2Y<sub>12</sub>transfected CHO-K1 cell membranes

pcDNA3 vector with cDNA for the human P2Y<sub>12</sub> receptor (Missouri S&T cDNA Resource Center, MI, USA) was transfected into Chinese Hamster Ovary Cells-K1 (CHO-K1) by using TransIT<sup>®</sup>-CHO Transfection kit (Mirus Bio, WI, USA). [ $^{35}$ S]GTP $\gamma$ S binding studies on competent P2Y<sub>12</sub>-transfected CHO-K1 cell membrane was performed essentially as described by JJJ Van Giezen *et al.*[8].

#### Results

## Farnesyl pyrophosphate potentiates LPA stimulated platelet shape change

FPP has previously have been reported to stimulate platelet shape change. However while our results show that FPP,  $30\mu M$ , clearly potentiated LPA stimulated shape change (\*P=0.0168, n=10), we were unable to show an effect of FPP alone (fig 1).

#### Farnesyl pyrophosphate antagonizes 2-MeSADP stimulated platelet aggregation

While confirming the results by Williams *et al.* [3] we noticed that FPP, except for the documented effect on shape change, also was exerting an inhibitory effect on platelet aggregation. In order to investigate this phenomenon we stimulated platelets with  $0.03\mu$ M

2-MeSADP in the presence of 30uM FPP. The results revealed a clear, dose dependent inhibition of aggregation (\*\*P<0.01, n=15) (fig 2A). However, the inhibitory effect was specific for ADP mediated aggregation as both TRAP (15uM), (<sup>n.s</sup>P=0.14, n=7) and epinephrine (1uM), (<sup>n.s</sup>P=0.054, n=10) stimulated aggregation were unaffected by 30µM FPP (fig 2B, C). In our next experiment we tested the other intermediates of the mevalonate pathway, related in chemical structure to FPP, for their ability to inhibit ADP stimulated aggregation only to find that FPP is the sole member with this kind of activity (\*\*P=0.01, n=6) (fig 3A). The FPP mediated inhibition of aggregation was dose-dependent resulting in 40 ( $\pm$ 5) % inhibition at 10  $\mu$ M (\*\*P=0.01, n=15) (fig 3B).

Farnesyl pyrophosphate decreases ADP stimulated up regulation of GPIIb/IIIa and P-selectin on platelets, hence inhibits ADP mediated attenuation of cAMP production, but has no effect on calcium mobilization.

In the next experiment we demonstrated that ADP stimulation of the platelet activation marker PAC-1 for GPIIb/IIIa activation was significant decreased in the presence of 30  $\mu$ M FPP (37 ± 5.3 vs. 16.1 ± 2.8, \*\*P=0.01, n=7). Also, the degranulation marker, P-selectin, was significantly decreased in the presence of

 $30\mu M$  FPP (16.4 ± 2.7 vs. 8.6 ± 1.1, \*P=0.05, n=6)

(fig 4A). These data correlates well with results on FPPs effect on ADP suppressed cAMP generation. The presence of 30  $\mu$ M FPP clearly reversed the ADP mediated attenuation of forskolin stimulated cAMP production in platelets (\*\*P=0.01, \*P=0.05 n=4) (fig 4B).

However when we loaded platelets with Fluo-4AM and tested the effect of FPP on ADP and thrombin stimulated calcium signalling, the results revealed no significance Ca<sup>2+</sup> statistical in mobilization, neither between platelets stimulated with 5uM ADP (<sup>n.s</sup>P=0.24, n=6) (fig 4 C:1) nor thrombin (0.5 U/ml)  $(^{n.s}P=0.46, n=6)$  in the presence or absence of 30uM FPP (fig 4 C:2).

# 2MeSADP stimulated [<sup>35</sup>S]<sub>7</sub>GTP platelet binding is antagonized by farnesyl pyrophosphate.

As the preceding results indicated the likely target to be  $P2Y_{12}$  we isolated platelet plasma membranes and investigated the ability of FPP to interfere with 2-MeSADP stimulated

 $[^{35}S]$ GTP $\gamma S$  binding. The presence of 60nM of the P2Y<sub>12</sub> specific antagonist ticagrelor right shifted the dose response curve thus confirming the identity of the target as P2Y<sub>12</sub>

EC50=17nM (2-MeSADP; VS. 2-MeSADP+60 nM ticagrelor;  $EC_{50}=0.42\mu M$ ) (fig 5A). FPP, at 6 uM, shifted the dose-response curve slightly to the left while, at the same time, decreasing the efficacy of 2-MeSADP (2-MeSADP; EC<sub>50</sub>=16nM vs. 2-MeSADP+30 µM FPP;  $EC_{50}=6.8nM$ ). The agonistic action of FPP was readily blocked by 10 µM ticagrelor P=0.05. (<sup>\*</sup>P=0.05. n=4) (fig 5B). However, when increasing the concentration of FPP, the antagonist properties became more obvious by simultaneously decreasing the efficacy and right shifting the dose-response curve of 2-MeSADP (fig 5C). EC<sub>20</sub> values were calculated and analyzed, (EC<sub>20(2-MeSADP</sub>): 0.95nM. EC<sub>20([FPP]</sub> 1\*10<sup>-5</sup> M): 4.5nM.  $EC_{20([FPP] 5*10^{-5}M)}$ : 39nM,  $EC_{20([FPP] 1*10^{-4}M)}$ : no data). The inhibitory effect mediated by FPP vs. 2-MeSADP on the platelet P2Y<sub>12</sub> receptor yielded an IC<sub>50</sub> value at 45 µM. The results indicate that FPP is a noncompetitive antagonist for the  $P2Y_{12}$ receptor with partial agonistic properties. The backbone structure of FPP is similar to the one of ADP. Both ligands consist of a hydrophobic and a hydrophilic part. The hydrophobic part presents a molecular variance. While FPPs hydrophobic part is made up of a 15-carbon chain, the corresponding part on the ADP nucleotide is composed of a ribose and adenine ring. However, the hydrophilic part is totally similar when comparing the two ligands as they both, structurally, are made up by negatively charged pyrophosphate groups (fig 6).

## Farnesyl pyrophosphate is a ligand to the $P2Y_{12}$ receptor.

We next repeated the  $[^{35}S]GTP\gamma S$  binding experiment using CHO cells stably expressing the  $P2Y_{12}$  receptor (fig 6). The results clearly confirms the identity of  $P2Y_{12}$  as the target by showing a dosedependent inhibition pattern, of 2-MeSADP stimulated [<sup>35</sup>S]GTPγS binding, that is very similar to that obtained with platelet membranes in the previous experiment and also confirms FPP as a non-competitive antagonist. The reason for the higher maximal response at 100 µM FPP, is likely due to the higher receptor density of the CHO cells in combination with the partial agonist nature of FPP. We repeated the calculation and analyze of EC<sub>20</sub> values from the CHOK1 binding assay, (EC<sub>20(2-MeSADP</sub>): 1nM, EC<sub>20([FPP] 1\*10</sub>  $_{M}^{5}$ : 2.8nM, EC<sub>20([FPP] 5\*10</sub><sup>-5</sup>M): 15nM,

 $EC_{20([FPP] 1*10}^{-4}M)$ : 21nM), and the result indicates the same thing, i.e increasing concentrations of FPP decreases the 2-MeSADP potency.

#### Docking of Farnesyl pyrophosphate to <u>a P2Y<sub>12</sub> receptor model</u>

## Structure of farnesyl pyrophosphate (FPP)

The sequences of all of the human adenosine and P2Y receptors were retrieved from the SWISS-PROT and TrEMBL database of the ExPASy Molecular Biology Server Bairoch et al. [9] and aligned using the CLUSTALW program Thompson et al. [10]. To verify the correct alignment of the highly conserved residues in the helices and to remove gaps within the transmembrane (TM) domains the automated alignment was manually adjusted. Because of the lack of sequence similarity in the aminoterminal domains and the carboxylterminal domains, the first 13 residues and the last 25 residues of the  $P2Y_{12}$  receptor sequence were excluded from the alignment and the modeling.

The crystallographic structure of the human A<sub>2A</sub> adenosine receptor (A<sub>2A</sub>AR), Jakkola et al. [11] was retrieved from the Protein Data Base, Berman et al. [12], (PDB ID: 3EML) and used as structural template for the construction of the  $P2Y_{12}$ receptor model by means of the Prime package; (Prime, vers. 2.1; Schrödinger LLC, NY, USA). The chemical correctness of the A2AAR structure was achieved with "Protein Preparation Wizard" the automated process in Maestro 9.0 (Maestro, vers. 9.0 Schrödinger LLC, NY, The T4-lysozyme USA). insertion replacing the third intracellular loop (IL3)

in the  $A_{2A}AR$  crystal structure was removed.

The highly conserved disulfide bridge between TM3 and the second extracellular loop (EL2) was built during the homology modelling stage based on the corresponding disulfide bridge in the A2AAR structure. Instead, the disulfide bridge between the N-terminal and the third extracellular loop (EL3), conserved within the P2Y family but absent in the A2AAR, was created afterward on the  $P2Y_{12}$  receptor model by means of the refine loops tool in Prime. During this stage, a cooperative loop sampling of the N-terminal region and EL3 was applied with a harmonic distance constraint between the thiol groups of Cvs17 in the N-terminal and Cys270 in the EL3, using a distance of 2.05 Å and a constant force of 350 kcal mol<sup>-1</sup> Å<sup>-2</sup>. The top ranked model from the loop refinement procedure was further inspected to assess the stereochemical quality and chosen for the molecular docking study.

All the ligands structures were sketched in Maestro and minimized with the Polak-Ribiere conjugate gradient (PRCG) minimization consisting of 1000 steps of CG until a gradient of 0.01 kJ/(mol Å<sup>2</sup>).

The molecular docking of the ligands was performed by means of the Induced Fit Docking (IFD) protocol as in the Schrödinger package Sherman et al. [13]. The docking site was defined according to the site-directed mutagenesis data [14, 15] and previous modeling results [16, 17]. A 26 Å x26 Å x26 Å box was centred on the ligands in the pocket. Briefly, in the first stage a docking of the ligands was performed in a rigid binding site with Glide using the SP (standard precision) procedure, (Glide, version 5.5, Schrödinger LLC, NY, USA), [5] using a van der Walls scaling of 0.5 for both receptor and ligands non polar atoms. For each ligand, the top 20 docking conformations were retained and subjected to the receptor sampling by means of the Refinement module in Prime. The Prime side-chain sampling and energy minimization were performed on all the residues within a 5 Å of the ligand in any of the 20 poses using the OPLS parameter and a surface Generalized Born implicit solvent model. The complexes within 30 kcal/mol of the minimum energy structure, ranked by Prime energy, were retained for the re-docking stage. In the final step, Glide with the XP (extra precision) scoring function and default parameters was used to re-dock all the ligands into their respective conformations produced by Prime. No constraints were used for all the docking calculations. The top scored model for each ligand was chosen as final binding conformation. The graphical pictures were made with the PYMOL program (Delano Scientific LLC, CA, USA).

FPP contains a hydrophobic 15-carbon chain and а negatively charged pyrophosphate group. It presents a very different scaffold from ADP or 2-MeSADP, typical agonists of the  $P2Y_{12}$ receptor. FPP shares a negatively charged phosphate moiety with ADP and 2-MeSADP but it has a hydrophobic 15carbon chain that is different from the either of the rings of the nucleotides. In order to understand the way FPP and 2-MeSADP bind to the same receptor and to find the common rational basis for the binding modes, a molecular modelling study was conducted. The three dimensional model of the P2Y12 receptor was built, based on the crystallographic structure of A2A adenosine receptor (PDB code: 3ELM), and the binding modes of 2-MeSADP and FPP were studied by means of flexible molecular docking.

The putative binding site of  $P2Y_{12}$  receptor was identified, based on the available sitedirected mutagenesis data Hoffmann *et al.* [15] and Cattaneo *et al.* [14] on previously published modeling studies by Costanzi *et al.* [16]. The recently published mutagenesis data confirmed the key role in the ligand binding of residues including  $Arg256^{6.55}$  in the sixth transmembrane domain (TM6) and Lys280<sup>7.35</sup> in TM7, as suggested by the docking model proposed by Costanzi et al. In our P2Y<sub>12</sub> receptor model with docked agonists these residues are directly involved in hydrogen bond interactions with the ligands. The binding pocket in our model is located in a pocket formed by residues of TM1, TM2, TM3, TM6, and TM7. In addition to the hydrophilic and charged residues like Lys280<sup>7.35</sup>, Arg256<sup>6.55</sup> and several aromatic residues including Tyr32<sup>1.39</sup> 105<sup>3.33</sup> Phe77<sup>2.57</sup>. Phe104<sup>3.32</sup>, Tyr Tyr259<sup>6.58</sup>, and (EL2), Phe177 hydrophobic residues including Leu284<sup>7.39</sup>, Ile81<sup>261</sup>, and Pro78<sup>2.58</sup>, and polar residues including Thr287<sup>7.42</sup> and Ser288<sup>7.43</sup> delineate the binding pocket of the  $P2Y_{12}$ receptor. Residues of the second extracellular loop (EL2), such as Ser176 and Leu178, delimit the upper part of the pocket.

From the docking results of 2-MeSADP in the  $P2Y_{12}$  receptor binding pocket, there is a crucial role of  $Arg256^{6.55}$  and  $Lys280^{7.35}$ in anchoring the diphosphate group of 2-MeSADP to the binding cavity. In addition, the hydroxyl group of Tyr2596.58 and the backbone amino group of Phe177 in EL2 are stabilizing the molecule from above with further hvdrogen bond interactions with the diphosphate group. In the adenine ring of 2-MeSADP, the amino group at position N<sup>6</sup> is stabilized by interactions with the side chain of Glu281<sup>7.36</sup>, while the N<sup>1</sup> atom interacts with the hydroxyl group of  $Tyr32^{1.39}$ . favourable interactions Other occur between 2-MeSADP and the residues Phe177 in EL2, Phe104<sup>3.32</sup>, Phe77<sup>2.57</sup>, Ile81<sup>2.61</sup>, Leu284<sup>7.39</sup>, Ser288<sup>7.43</sup>, and Thr287<sup>7.42</sup> as shown in fig 9 A.B.

From the docking results of FPP in the  $P2Y_{12}$  receptor binding site, the pyrophosphate group binds, as expected, in a similar way to the diphosphate moiety of

2-MeSADP (fig 8A.B.C). Strong electrostatic interactions occur between the diphosphate moiety of FPP and the side chains groups of Arg256<sup>6.55</sup> and Lys280<sup>7.35</sup>. The hydroxyl group of Ser176 and the backbone groups of Leu178 and Phe177, all in EL2, stabilize the pyrophosphate group of FPP in the pocket. The binding mode of FPP showed a conformation with an extended orientation of the flexible chain of FPP. The hydrophobic nature of the isoprenoid chain of FPP would be expected to lead to a more folded conformation. The 15-carbon flexible farnesyl group can assume both a trans or gauche disposition about the C<sub>4</sub>-C<sub>5</sub> bond of the chain. The trans conformation leads to an extended form of the chain while the gauche conformation leads to a folded form of FPP. From the docking results of FPP in P2Y<sub>12</sub> binding pocket, the compound adopts a trans conformation about the C4-C5 bond with an extended isoprenoid chain. An extended conformation for the farnesyl chain of FPP both in solution and in a protein-bound conformation was already observed in experimental several other and computational studies on isoprenoid derivatives, Cui et al [18] and Zahn et al. [19, 20]. In the  $P2Y_{12}$  receptor binding pocket, the aromatic and hydrophobic side chains of residues such as  $Tyr32^{1.39}$ , Phe $77^{2.57}$ , Phe $104^{3.32}$ , and Leu $284^{7.39}$  make optimal contacts with the isoprenoid chain creating a favourable environment to embed the FPP.

Fig 9C and illustrates a superposition of the best docking poses of FPP and 2-MeSADP shows the steric and electronic overlap between the two molecules. The  $\alpha$ phosphate group of FPP is superimposed on the  $\beta$ -phosphate group of 2-MeSADP, but the diphosphate groups of both the compounds share the same strong electrostatic interactions with the residues in the top part of the  $P2Y_{12}$  receptor binding pocket. Moreover, the farnesyl chain of FPP is well overlapped with the adenine ring and the thiomethyl group at

the 2 position of 2-MeSADP. The hydrophilic nitrogen atom N<sup>1</sup> of the adenine ring is reaching out for the hydroxyl group of Tyr32<sup>1.39</sup> creating a hydrogen bond interaction, while the terminal of the isoprenoid chain of FPP is oriented deep under the aromatic ring of  $Tvr32^{1.39}$ . The possible involvement of  $Tvr32^{1.39}$  in the binding of nucleotide ligands was previously suggested by Costanzi et al [16]. The FPP chain is reaching the bottom of the pocket, close to the residues Leu $35^{1.42}$ , Pro $78^{2.58}$ , and Ser288<sup>7.43</sup>. In order to maintain an extended conformation of the isoprenyl chain, the pyrophosphate group can not bind as deep as the diphosphate group of 2-MeSADP, as shown by the superposition between the docking poses of FPP and 2-MeSADP, even if the interaction between the diphosphate group and the positively charged residues in the binding pocket are conserved for both the molecules.

#### Discussion

In the present study we demonstrate that FPP, apart from being an intermediate in the mevalonate pathway, is an endogenous non-competitive antagonist of the P2Y12 receptor acting as an inhibitor of ADP induced platelet aggregation.

The mevalonate pathway is a metabolic pathway of great importance to all eukarvotic cells. Although the isoprenoid metabolites of this pathway, FPP, GGPP, DMPP and IPP are mainly known as precursors in the biosynthetic route of cholesterol or to mediate the membrane association of certain GTPases, Goldstein et al [1] there have recently been several reports that indicates alternative functions. Farnesyl pyrophosphate was reported to serve as an antagonist at the classical LPA2 and LPA3 receptors while being an agonist on the atypical LPA<sub>4</sub>, LPA<sub>5</sub> and LPA<sub>6</sub>, Lee et al. [21]. Furthermore, FPP has also been described by Bang et al. [22] as an agonist of the pain producing TRPV3 channel while GGPP is known to antagonize the liver X receptor (LXR), Gan et al. [23] In a previous study, using gene array screening, we identified the message of the recently deorphanized LPA receptors LPA4 and LPA5 in human platelet mRNA, Amisten et al. [24]. As these receptors both have been described as receptors for FPP by Williams et al. [3] we tested the effect of this compound on platelet activation. Although we were able confirm the findings previous to investigated by Williams et al. [3] who reported an effect of FPP on platelet shape change mediated by LPA<sub>5</sub>, we noticed that the major effect was antagonistic rather than agonistic and specifically antagonistic to ADP stimulated aggregation, as both aggregation triggered by TRAP or by epinephrine were unaffected. FPP had no effect on 2-MeSADP stimulated calcium mobilisation in platelets thus ruling out the other ADP receptor, P2Y<sub>1</sub>, as a target. However, both the degranulation marker Pselectin and the adhesion integrin, GPIIb/IIIa, was attenuated in the presence of FPP. These results correlates well with results from cAMP measurement which showed that FPP repressed ADP induced decrease in cAMP generation. A recent report states that between 60 % - 90 % of the platelet  $P2Y_{12}$  receptor sites need to be order blocked in for the VASP phophorylation attenuation to be detectable, HM Judge et al [25] and Schumacher et al. [26]. However, when we studied 2-MeSADP stimulated [35S]GTPyS binding in platelet membranes the action of FPP. above 10 was μM, clearly antagonistic affecting both efficacy and potency thus showing the typical hallmarks non-competitive antagonism. of At concentrations below 6 uM, FPP [<sup>35</sup>S]GTPγS independently stimulated binding rather than blocking it. This effect could be completely reversed by ticagrelor thus indicating  $P2Y_{12}$  as the main target. In order to confirm the target identity we tested the ability of FPP to compete with 2-

MeSADP on P2Y12 receptors expressed in CHO cells. The results substantiated the platelet results as FPP was able to antagonize the effects of 2-MeSADP [<sup>35</sup>S]GTPγS stimulated binding. The antagonistic response to FPP, in P2Y<sub>12</sub>-CHO cells was slightly less than for platelet membranes and the most likely explanation for this is that FPP being a partial agonist, is more dependent on receptor density. Thus in the recombinant cell system with a high receptor density an affinity-driven partial agonist will activate a higher proportion of receptors.

The affinity of FPP for P2Y<sub>12</sub> is understandable, partly because of the structural similarity between ADP and FPP but also since the receptors LPA<sub>5</sub> and LPA<sub>6</sub>, both phylogenetically related to P2Y12, previously been demonstrated to bind FPP. As both LPA<sub>5</sub>, LPA<sub>6</sub> and P2Y<sub>12</sub> belong to the purinergic cluster of GPCRs, Fredriksson et al. [27] and sharing a high degree of general structural similarity, they are also likely to have similarities in the receptor binding pocket. Indeed. а chemogenomic evaluation of the transmembrane ligand binding cavity of GPCRs placed LPA<sub>5</sub> (GPR92), LPA<sub>6</sub>  $(P2Y_5)$  and  $P2Y_{12}$  in the same group, Surgand et al. [28]. To further test the validity of our results we evaluated the ability of farnesyl pyrophosphate to dock into the binding cavity of the human P2Y<sub>12</sub> receptor.

From the docking poses of 2-MeSADP and FPP, we could deduct that the positively charged residues in the upper part of the binding cavity of the P2Y<sub>12</sub> receptor are essential for the binding of the negatively charged diphosphate groups. The docking modes of FPP and

2-MeSADP nicely superpose with the side chain at position 2 of 2-MeSADP and the terminal part of the farnesyl chain of FPP pointing both toward a deep region of the  $P2Y_{12}$  binding pocket formed by residues of TM1, TM2, and TM7. The aromatic and hydrophobic residues in the  $P2Y_{12}$  cavity

create a good environment for both of the compounds. The side chains of the residues at the bottom of the binding pocket are well adjusting around the different side chains of the ligands. In particular, in the complex with 2-MeSADP the aromatic ring of  $Tyr32^{1.39}$  and the polar chain of Ser288<sup>7.43</sup> are oriented more into the pocket closing around the short 2methylthio chain at position 2 compared to the pocket of the complex with FPP, where Tyr32<sup>1.39</sup> ring is slightly higher and the hydroxyl group of Ser288<sup>7.42</sup> is pointing away from the pocket creating more room for the longer isoprenyl chain of FPP and also reducing the polarity of the cavity. To summarise: as the docking modes of FPP and ADP are superimposable there are reason to believe that they reflect the function meaning that, based on the docking pose, it is more likely to expect FPP to be a partial agonist than a pure antagonist. This is in agreement with our finding of agonistic effects in lower concentrations of FPP  $[^{35}S]$ GTP $\gamma$ S binding analysis in platelet membrane.

Although FPP have been reported by Taggart et al [2] to be present in the circulation only a single report describes physiological circumstances affecting FPP plasma levels showing that fasting and feeding cause different diurnal variations in do FPP plasma levels. To understand a possible physiological role for FPP as platelet regulator, we need to know the concentrations in different plasma physiological and patophysiological situations, such as coronary ischemia and inside a thrombus. In low concentrations FPP could have a prothrombotic effect both by stimulating LPA induced shape change via LPA receptors or by partial agonism at P2Y<sub>12</sub> receptors. However, at higher concentrations (micromolar), FPP is antithrombotic by blocking the fundamentally important positive feedback loop of ADP stimulating P2Y<sub>12</sub> receptors. P2Y<sub>12</sub> blockers are among the most used drugs for treatment of acute coronary

syndromes. Clopidogrel has a variable effect and patients with diabetes or CYP2C19 variants are commonly poor responders. The more potent  $P2Y_{12}$  blockers prasugrel and ticagrelor has been shown to be superior to clopidogrel in preventing myocardial infarction, stent thrombosis and mortality, Michelson *et al.* [29] and Wallentin *et al* [30]. FPP could be seen as everyman's own endogenous  $P2Y_{12}$  blocker that may balance the strong pro-aggregatory effects of ADP.

Another interesting aspect is the widely used statins, which are inhibitors of the mevalonate pathway and thereby FPP synthesis. Could this be one explanation for the pleiotrophic effects of statins? In the MIRACLE and ARMYDA-ACS studies, the incidence of myocardial infarction was reduced very early and it has been argued that this is to short time for cholesterol lowering to reduce plaque burden or even stabilising the plaque [31. 32]. Based on this pleiotrophic effects have been suggested to explain the early effects of statins [33]. Statins have been shown to reduce platelet aggregation. If changes in FPP synthesis contribute to these effects needs to be examined.

In conclusion, FPP is a competitive antagonist of ADP induced  $P2Y_{12}$  mediated platelet aggregation. It could be an endogenous antithrombotic factor modulating the strong platelet aggregating effects of ADP in a similar way as we use clopidogrel, prasugrel or ticagrelor in ischemic heart disease treatment.

#### References

- Goldstein, J.L. and M.S. Brown, *Regulation of the mevalonate pathway.* Nature, 1990. **343**(6257): p. 425-30.
- McTaggart, S.J., *Isoprenylated proteins*. Cell Mol Life Sci, 2006. 63(3): p. 255-67.

- 3. Williams, J.R., et al., Unique ligand selectivity of the GPR92/LPA5 lysophosphatidate receptor indicates role in human platelet activation. J Biol Chem, 2009. **284**(25): p. 17304-19.
- Hogberg, C., D. Erlinge, and O.O. Braun, Mild hypothermia does not attenuate platelet aggregation and may even increase ADP-stimulated platelet aggregation after clopidogrel treatment. Thromb J, 2009. 7: p. 2.
- Cazenave, J.P., et al., Preparation of washed platelet suspensions from human and rodent blood. Methods Mol Biol, 2004. 272: p. 13-28.
- Liu, E.C. and L.M. Abell, Development and validation of a platelet calcium flux assay using a fluorescent imaging plate reader. Anal Biochem, 2006. 357(2): p. 216-24.
- Vasiljev, K.S., A. Uri, and J.T. 7. Laitinen. 2-Alkvlthio-substituted platelet P2Y12 receptor antagonists reveal pharmacological identity between the rat brain Gi-linked ADP receptors and *P2Y12*. Neuropharmacology, 2003. 45(1): p. 145-54.
- JJ, V.A.N.G., et al., *Ticagrelor* binds to human P2Y(12) independently from ADP but antagonizes ADP-induced receptor signaling and platelet aggregation. J Thromb Haemost, 2009. 7(9): p. 1556-65.
- 9. Bairoch, A. and R. Apweiler, *The SWISS-PROT* protein sequence database and its supplement *TrEMBL* in 2000. Nucleic Acids Res, 2000. **28**(1): p. 45-8.
- 10. Thompson, J.D., D.G. Higgins, and T.J. Gibson. CLUSTAL W: improving the sensitivity ofprogressive multiple sequence through alignment sequence

weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res, 1994. **22**(22): p. 4673-80.

- 11. Jaakola, V.P., et al., *The 2.6* angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science, 2008. **322**(5905): p. 1211-7.
- 12. Berman, H.M., et al., *The Protein Data Bank*. Nucleic Acids Res, 2000. **28**(1): p. 235-42.
- Sherman, W., et al., Novel procedure for modeling ligand/receptor induced fit effects. J Med Chem, 2006. 49(2): p. 534-53.
- 14. Cattaneo, M., et al., Molecular bases of defective signal transduction in the platelet P2Y12 receptor of a patient with congenital bleeding. Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1978-83.
- 15. Hoffmann, K., et al., Involvement of basic amino acid residues in transmembrane regions 6 and 7 in agonist and antagonist recognition of the human platelet P2Y(12)receptor, in Biochem Pharmacol. 2008. p. 1201-13.
- Costanzi, S., et al., Architecture of P2Y nucleotide receptors: structural comparison based on sequence analysis, mutagenesis, and homology modeling. J Med Chem, 2004. 47(22): p. 5393-404.
- Ivanov, A.A., S. Costanzi, and K.A. Jacobson, *Defining the nucleotide binding sites of P2Y receptors using rhodopsin-based homology modeling*. J Comput Aided Mol Des, 2006. 20(7-8): p. 417-26.
- 18. Cui, G. and K.M. Merz, Jr., Computational studies of the farnesyltransferase ternary complex part II: the conformational activation of farnesyldiphosphate.

Biochemistry, 2007. **46**(43): p. 12375-81.

- Zahn, T.J., M.B. Ksebati, and R.A. Gibbs, Synthesis and conformational analysis of di-<sup>13</sup>C-labeled farnesyl diphosphate analogs. Tetrahedron Letters, 1998. 39(23): p. 3991-3994.
- Zahn, T.J. and M. Eilers, Evaluation of Isoprenoid Conformation in Solution and in the Active Site of Protein-Farnesyl. Journal of the American Chemical Society, 2000. 122(30): p. 7153-7165.
- Lee, M., et al., P2Y5 is a G(alpha)i, G(alpha)12/13 G protein-coupled receptor activated by lysophosphatidic acid that reduces intestinal cell adhesion. Am J Physiol Gastrointest Liver Physiol, 2009. 297(4): p. G641-54.
- Bang, S., et al., Farnesyl pyrophosphate is a novel pain-producing molecule via specific activation of TRPV3. J Biol Chem. 285(25): p. 19362-71.
- 23. Gan, X., et al., *Dual mechanisms of ABCA1* regulation by geranylgeranyl pyrophosphate. J Biol Chem, 2001. **276**(52): p. 48702-8.
- Amisten, S., et al., Gene expression profiling for the identification of Gprotein coupled receptors in human platelets. Thromb Res, 2008. 122(1): p. 47-57.
- 25. Judge, H.M., et al., *Relationship* between degree of P2Y12 receptor blockade and inhibition of P2Y12mediated platelet function. Thromb Haemost. **103**(6): p. 1210-7.
- Schumacher, W.A., et al., Biomarker optimization to track the antithrombotic and hemostatic effects of clopidogrel in rats. J Pharmacol Exp Ther, 2007. 322(1): p. 369-77.

- Fredriksson, R., et al., The Gprotein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol, 2003.
  63(6): p. 1256-72.
- 28. Surgand, J.S., et al., *A* chemogenomic analysis of the transmembrane binding cavity of human *G*-protein-coupled receptors. Proteins, 2006. **62**(2): p. 509-38.
- Michelson, A.D., et al., *Pharmacodynamic assessment of platelet inhibition by prasugrel vs. clopidogrel in the TRITON-TIMI 38 trial.* Eur Heart J, 2009. **30**(14): p. 1753-63.
- Wallentin, L., et al., *Ticagrelor* versus clopidogrel in patients with acute coronary syndromes. N Engl J Med, 2009. 361(11): p. 1045-57.
- 31. Schwartz, G.G., et al., *Effects of* atorvastatin on early recurrent ischemic events in acute coronary syndromes: the MIRACL study: a randomized controlled trial. Jama, 2001. **285**(13): p. 1711-8.
- 32. Patti, G., et al., Atorvastatin pretreatment improves outcomes in patients with acute coronarv syndromes undergoing earlv percutaneous coronary intervention: results of the ARMYDA-ACS randomized trial. J Am Coll Cardiol, 2007. 49(12): p. 1272-8.
- Labios, M., et al., Effect of atorvastatin upon platelet activation in hypercholesterolemia, evaluated by flow cytometry. Thromb Res, 2005. 115(4): p. 263-70.

Figure 1.



Fig 1 LTA with PRP.  $30\mu M$  FPP has an additive effect on  $10\mu M$  LPA mediated platelet shape/change activation. Student's *t*-test: \*P<0.05, n=10.

Figure 2.



**Fig 2.** LTA with PRP. (A) FPP (30 $\mu$ M) shows an inhibitory effect on 2-MeSADP (0.03 $\mu$ M) mediated platelet activation, Student's *t*-test: <sup>\*\*</sup>P<0.01, n=15. (B) FPP (30 $\mu$ M) has no inhibitory effect on TRAP (10 $\mu$ M), Student's *t*-test: <sup>n.s</sup>P=0.077, n=7. (C) FPP (30 $\mu$ M) has no inhibitory effect on epinephrine (1 $\mu$ M), Student's *t*-test: <sup>n.s</sup>P=0.054, n=15. Each of bargraph accompanied by a representing tracer.



Figure 3A and 3B.

Fig 3A. LTA on washed platelets. Screening for the inhibitory capacity of intermediates present in the mevalonatic pathway ( $30\mu$ M) vs. 2-MeSADP ( $0.04\mu$ M). Only FPP had statistic significance, One-way ANOVA analysis: \*\*P<0.01, n=6.



**Fig 3B**. LTA on PRP. Bar graph representing the antagonistic effect of FPP on  $P2Y_{12}$  in an dos-responding manner, One-way ANOVA analysis:<sup>\*\*</sup>P<0.01, n=15.

#### Figure 4A and 4B.



**Fig 4A.** Flow cytometric study on platelet GPIIb/IIIa and P-Selectin activation, shoving the decreasing effect of ADP in the presence of FPP ( $30\mu M$ ) compared to ADP. Student's *t*-test:<sup>\*\*\*</sup>P=0.001, \*P=0.05, n=15).



**Fig 4B.** Bar graph representing cAMP measuring on platelets. Statistical significance were seen between Forskolin (10 $\mu$ M) treated platelets vs. platelets incubated with 2-MeSADP (1\*10<sup>-9</sup>M), One-way ANOVA analysis: \*\*P<0.01. A significance in cAMP production were also seen between platelets treated with 2-MeSADP and Forskoline vs. platelets also pre-treated with FPP (30 $\mu$ M), One-way ANOVA analysis: \*P<0.05, n=4.

Figure 4C



**Fig 4C.** Calcium flux on platelets. Tracer 1. representing the non-significant relationship between 2-MeSADP treated platelets ( $\circ$ ) vs. platelets pre-treated with FPP (30 $\mu$ M) ( $\bullet$ ), Student's *t*-test: <sup>n.s</sup>P=0.24, n=6. Tracer 2. representing the non-significant relationship between platelets activated by thrombin (0.5U/ml) ( $\circ$ )vs. platelets, pre-treated with FPP (30 $\mu$ M) ( $\bullet$ ), Student's *t*-test: <sup>n.s</sup>P=0.46, n=6.





**Fig 5A.** [<sup>35</sup>S]GTP $\gamma$ S binding on platelet membranes, identifying the P2Y<sub>12</sub> receptor. When adding the P2Y<sub>12</sub>-receptor blocker ticagrelor, there becomes a shift towards the right in the dos-response curve. Platelet membranes treated with increasing concentrations of 2-MeSADP, ( $\circ$ ), receive an EC<sub>50</sub> value at 17nM, whereas platelet membranes pre-incubated with ticagrelor (60nM), ( $\bullet$ ), increase the EC<sub>50</sub> value to 0.4  $\mu$ M. Bar graph representing the blocking effect of ticagrelor (1 resp 10 $\mu$ M) on 2-MeSADP mediated P2Y<sub>12</sub> receptor on platelet membranes (One-way ANOVA analysis: <sup>\*\*</sup>P<0.01, <sup>\*\*\*</sup>P<0.001, n=4).





**Fig 5B.** Upper figure:  $[^{35}S]$ GTPγS binding on platelet membranes identifying FPP (6μM) having an endogenous antagonist effect on the 2-MeSADP stimulated P2Y<sub>12</sub> receptor. FPP shift the dose-response curve to the left, i.e. increase the potency of 2-MeSADP. Platelet membranes treated with increasing concentrations of 2-MeSADP, ( $^{\circ}$ ), receive an EC<sub>50</sub> value at 16nM, whereas platelet membranes pre-incubated with FPP (30µM), ( $^{\bullet}$ ), increase the EC<sub>50</sub> value to 7nM. Lower figure: Bar graph representing the attenuating effect of 1µM FPP on platelet membrane protein. This effect is suppressed by 10µM of the P2Y<sub>12</sub> receptor blocker ticagrelor which indicates that FPP interacts with P2Y<sub>12</sub>. (One-way ANOVA analysis: \*\*P<0.01, \*\*\*P<0.001, n=4).





**Fig 5C.** Upper figure: [<sup>35</sup>S]GTP $\gamma$ S binding study on platelet membrane to investigate FPPs antagonistic/agonistic features in a dose-responding manner, in presence of increasing concentrations of 2-MeSADP. 2-MeSADP (control), EC<sub>20</sub>=0.95nM (**•**), 2-MeSADP with; [FPP] 10 $\mu$ M, EC<sub>20</sub>=4.9nM (**□**), [FPP] 50 $\mu$ M, EC<sub>20</sub>=39nM (**•**), and [FPP] 100 $\mu$ M, EC<sub>20</sub>=n.d ( $\circ$ ). There is a right shift in the curve unison with increasing concentrations of FPP. A right shift in the dose-response curve and a decrease in potency and efficacy relative to the increasing concentrations of FPP. Lower figure: The analyzed IC<sub>50</sub>-value on 45 $\mu$ M is calculated from dose-response data at [2-MeSADP]=1 $\mu$ M.









Fig 6. Molecular backbone structures of FPP and 2-MeSADP.

Figure 7.



**Fig 7.** [<sup>35</sup>S]GTP $\gamma$ S binding study on P2Y<sub>12</sub>-transfected CHOK1-cells, to study FPPs antagonistic/agonistic features in an dose-responding manner, in presence of increasing concentrations of 2-MeSADP. 2-MeSADP (control), EC<sub>20</sub>=1nM (**■**), 2-MeSADP with; [FPP] 10 $\mu$ M, EC<sub>20</sub>=2.8nM (**□**), [FPP] 50 $\mu$ M, EC<sub>20</sub>=15nM (**●**), and [FPP] 100 $\mu$ M, EC<sub>20</sub>=21nM (**○**). Like the case of platelet membrane protein, there is a right shift in the curve unison with increasing concentrations of FPP. A right shift in the dose-response curve and a decrease in potency and efficacy, relative to increasing concentrations of FPP.





**Fig 8.** Fig 8A and 8B visualizes the computerized calculated docking pose of FPP in the binding cavity of the  $P2Y_{12}$  receptor model. The FPP (depicted as ball and stick with yellow carbon atoms) is anchored in the pocket by strong electrostatic interactions between the charged and hydrophilic side chains and the negatively charged diphosphate group. The isoprenoid chain of FPP is favourably embedded by aromatic and hydrophobic residues of the  $P2Y_{12}$  receptor. In Fig 8B, the residues at the back of the pocket of  $P2Y_{12}$  is represented as surface colored by electrostatic potential (red for the partially negative charges, blue for the partially positive charges, and white for the neutral atoms). Fig 8C illustrates the complex shown from a top view, highlighting the orientation of the FPP chain deep in the pocket toward TM1 and TM2. For clarity only the residues interacting with the pyrophosphate are visible.





**Fig 9.** Fig 9A: binding mode of 2-MeSADP after docking in  $P2Y_{12}$  receptor binding site. 2-MeSADP is shown in a ball and stick representation with green carbon atoms. The diphosphate group of 2-MeSADP has the same interactions with the residues of the  $P2Y_{12}$  receptor shown for FPP in Fig. 8A. Differently from the hydrophobic chain of FPP, the more hydrophilic adenosine group of 2-MeSADP is anchored in the pocket by other electrostatic interactions with the residues in the pocket. The exocyclic amino group at the N<sup>6</sup> position of the adenine ring is interacting with the carboxyl group of E281, and the ring N<sup>1</sup> atom is interacting with the hydroxyl group of Y32. In Fig 9B, the residues in the binding pocket are represented with a surface. Fig 9C visualizes the calculated superposition of the docking poses of FPP (in yellow) and 2-MeSADP (in green) in the binding pocket of the P2Y<sub>12</sub> receptor. Only the ribbon representing the backbone of the receptor is shown.