Studies of platelet signalling and endothelial cell responses
using unique synthetic drugs

Somehow, we'll find it. The balance between whom we wish to be and whom we need to be. But for now, we simply have to be satisfied with who we are.
- Brandon Sanderson, The Hero of Ages

I dedicate this thesis to my family,
thank you for everything.

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Abstract

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Haemostasis is a complex and tightly regulated process which protects us from bleeding. Platelets are essential for maintained haemostasis. Under normal conditions platelets are calmed by antithrombotic substances release by the endothelium. During vascular injury, the platelets will activate and form a haemostatic plug to prevent bleeding. Inflammatory processes like atherosclerosis can disturb the haemostatic balance and lead to severe consequences like myocardial infarction and stroke. Inhibition of platelets and coagulation are common treatments to prevent unwanted blood clot formation. There is a great need for increased knowledge on the mechanisms of thrombosis and characterisation of new substances with possible therapeutic potential. This thesis used unique synthetic drugs to study platelet signalling and endothelial responses.
Paper I showed that both sulfated polysaccharides from seaweed and synthetic glycopolymers which mimic their chemical properties caused platelet activation.
Paper II elucidated the molecular mechanism underlying platelet activation by sulfated glycopolymers and polysaccharides. We found that human platelet activation took place via the Platelet endothelial aggregation receptor 1 (PEAR1), while mouse platelet activation was mainly via C-type lectin-like receptor 2. Aggregation was supported by Glycoprotein Ibα in both species.
Paper III showed the effect of synthetic glycopolymers and natural polysaccharides on cultured human endothelial cells. We found that both the glycopolymers and polysaccharides caused a proinflammatory response after 24h.
In **Paper IV**, the effect of a synthetic purine analogue with a nitrate ester motif was studied. We found that the purine analogue reduced platelet functions by inhibiting Rho-associated protein kinase (ROCK).
This thesis describes unique synthetic drugs that can be used for further studies of the mechanisms underlying the biological processes of thrombosis and inflammation. The synthetic glycopolymers can be used to further elucidate the physiological role of PEAR1, a potential future therapeutic target.

Keywords: Haemostasis, glycopolymers, purine analogue, PEAR1, GPIbα, CLEC-2, inflammation, ROCK.

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Populärvetenskaplig sammanfattning

Trombocyter, i vardagligt tal kallade blodplättar, är små cellfragment som cirkulerar i kroppens blodkärl. Trombocyterna bildas i benmärgen och deras huvudsakliga uppgift är att förhindra blödning. När trombocyterna kommer i kontakt med en skada i blodkärlet klumpas de samman och stoppa blödningen. Deras förmåga att stoppa blödning är livsviktigt för människan och regleras på många olika sätt.

Vid sjukdom, som åderförkalkning, så kan trombocyterna klumpas samman för mycket och på felaktig plats vilket gör att blodkärlet täpps till. När det sker så stryps syretillförseln till vävnaden, vilket kan leda till hjärtinfarkt eller stroke, vilket är några av världens vanligaste dödsorsaker.

Om man har ökad risk för att insjukna i hjärtinfarkt eller stroke så kan man behandlas med så kallade trombocythämmare. Trombocythämmare är mediciner som stoppar trombocyter från att aktiveras och klumpas samman. Tyvärr är behandlingar mot blodpropp ett tveeggat svärd. När trombocyterna är hämmade och inte kan klumpas samman så kan man istället råka ut för allvarlig blödning. Behovet av att hitta nya effektiva behandlingar för att motverka blodproppar och dess följdkonsekvenser är därför stort. I den bästa av världar vill vi hitta en medicin som motverkar blodproppar och minskar risken blödning.

Vanliga behandlingar mot blodproppar är mediciner som direkt påverkar trombocyterna, eller andra proteiner involverade i blodproppsbildning. Idag är heparin, en sorts naturligt förekommande kolhydrat, en vanlig medicin för att minska risken för blodpropp. Den används till exempel efter kejsarsnitt, vissa operationer, eller i hjärt-och-lung-maskiner. I denna avhandling undersöker jag hur andra naturligt förekommande kolhydrater från sjögräs, så kallade ”fukoidan”, och laboratorietillverkade versioner av dessa kolhydrater, också kallade ”glykopolymerer”, påverkar trombocyter.

Arbete I undersöker hur naturligt förekommande fukoidan och konstgjorda glykopolymerer påverkar trombocyter. Där upptäcktes att både fukoidan och glykopolymerer, till skillnad från heparin, aktiverar trombocyter. Vi undersökte hur längden på glykopolymererna påverkar trombocyt-aktivering och upptäckte att kedjor på 13 socker räcker för att orsaka aktivering av trombocyter.

Arbete II undersökte hur aktiveringen av fukoidan och glykopolymerer går till. Vi upptäckte att båda två aktiverar mänskliga trombocyter genom ett protein på trombocytens yta som kallas ”Platelet endothelial aggregation receptor 1” (PEAR1). I studien upptäckte vi också att dessa substanser aktiverar mustrombocyter och människotrombocyter på olika sätt. I möss aktiveras trombocyterna istället via ”C-type lectin-like receptor 2” (CLEC-2). I båda arterna behövs också ”Glykoprotein Ibα” (GPIbα) för att aktiveringen ska ske fullständigt.

Proteinet PEAR1 finns också i blodkärlets vägg, på så kallade endotelceller. I **Arbete III** undersökte vi därför hur fukoidan och glykopolymerer påverkar endotelceller som är odlade på laboratoriet. Vi såg att både glykopolymerer, fukoidan och en annan typ av kolhydrat bildar ett inflammationssvar hos endotelcellerna. När endotelcellerna utsätts för kolhydraterna och glykopolymererna så bildas ett protein som heter ”C-X-C motif chemokine 11” (CXCL11). Det proteinet frisätts normalt för att hjälpa immunceller hitta rätt om det uppstår inflammation någonstans i kroppen. Här finns det dock mer att göra, rollen av PEAR1 i det inflammationssvaret vi såg måste fortfarande klargöras.

I **Arbete IV** undersöker vi hur en annan typ av konstgjorda molekyl, en så kallad purin-analog, påverkar trombocyternas förmåga att klumpa samman. Denna molekyl har tidigare visat sig vara effektiv för att förhindra vävnadsskada vid hjärtinfarkt i försöksdjur. I **Arbete IV** beskriver vi hur purin-analogen påverkar mänskliga trombocyter. Vi upptäckte vi att purin-analogen stoppar tromboycternas förmåga att bilda aggregat genom att blockera ”Rho-associated protein kinase” (ROCK), ett protein som finns på trombocyternas insida. Tyvärr behövdes ganska stora mängder av purin-analogen för att den skulle ha tillräckligt hög effekt. Vi hoppas att i framtiden kunna förbättra purin-analogens kemiska struktur för att göra den mer effektiv.

Sammantaget så har denna avhandling visat att konstgjorda molekyler, som glykopolymerer och purin-analoger, är unika verktyg för att studera hur trombocyter aktiveras och endotelceller påverkas. Genom att vidare studera vad proteinet PEAR1 har för roll i människans fysiologi så hoppas vi att man kan öka kunskapen om hur PEAR1 påverkar människokroppen, och specifikt hur blodproppar och inflammationssvar bildas. Purin-analogen hoppas vi kunna förbättra. Förändringar av molekylens kemiska struktur kan möjligen göra den mer effektiv. Genom ökad förståelse om blodproppsbildningen och inflammationens grundläggande biologi hoppas vi att man i framtiden ska kunna tillverka nya mediciner för att behandla åderförkalkning och minska risken för hjärtinfarkt och stroke, de vanligaste dödsorsakerna i världen.

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Mattias Tengdelius, Caroline Kardeby, Knut Fälker, May Griffith, Peter Påhlsson, Peter Konradsson, and Magnus Grenegård.
Macromolecular Bioscience 2017 Feb;17(2) Epub 2016 Sep 12.

II. **Synthetic glycopolymers and natural fucoidans cause human platelet aggregation via PEAR1 and GPIbα.**
Caroline Kardeby, Knut Fälker, Elizabeth J. Haining, Maarten Criel, Madelene Lindkvist, Ruben Barroso, Peter Påhlsson, Liza U. Ljungberg, Mattias Tengdelius, G. Ed Rainger, Stephanie Watson, Johannes A. Eble, Mark F. Hoylaerts, Jonas Emsley, Peter Konradsson, Steve P. Watson, Yi Sun, and Magnus Grenegård.
Blood Advances, 2019 Feb 12;3(3):275-287.

III. **Sulfated glycopolymers and polysaccharides regulate inflammation-related proteins in human vascular endothelial cells.**
Caroline Kardeby, Allan Sirsjö, Liza U. Ljungberg, and Magnus Grenegård.
Manuscript.

IV. **A novel purine analogue bearing nitrate ester prevents platelet activation by ROCK activity inhibition.**
Caroline Kardeby, Geena V. Paramel, Dimitra Pournara, Theano Fotopoulou, Allan Sirsjö, Maria Koufaki, Karin Fransén, and Magnus Grenegård.
Manuscript, submitted.

Additional studies

Studies not included in this thesis:

IL-1α Counteract TGF-β Regulated Genes and Pathways in Human Fibroblasts.
Anita Koskela von Sydow, Chris Janbaz, Caroline Kardeby, Dirk Repsilber, and Mikael Ivarsson.
Journal of Cellular Biochemistry, 2016 Jul;117(7):1622-32 Epub 2015 Dec 28.

Adrenoceptor α2A signalling countervails the taming effects of synchronous cyclic nucleotide-elevation on thrombin-induced human platelet activation and aggregation.
Knut Fälker, Liza U. Ljungberg, Caroline Kardeby, Madelene Lindkvist, Allan Sirsjö, and Magnus Grenegård.
Cellular Signalling, 2019 Jul;59:96-109

List of Abbreviations

4G10	Pan-phosphotyrosine antibody
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Akt	Protein kinase B
ANOVA	Analysis of variance
AVEXIS	AVidity-based Extracellular protein Interaction Screen
cAMP	Cyclic adenosine monophosphate
CCL2	C-C motif chemokine ligand 2
CD42b	Cluster of differentiation 42b, also known as GPIbα
CD62P	P-selectin
cGMP	Cyclic guanosine monophosphate
Clec1b	C-type lectin domain family 1 member B, known as CLEC-2
CLEC-2	C-type lectin-like receptor 2
CPBDT	2-Cyano-2-propyl benzodithioate
CTA	Chain-transfer agent
CXCL11	C-X-C motif chemokine 11
DNA	Deoxyribonucleic acid
dNTPs	deoxy nucleoside triphosphates
ELISA	Enzyme-linked immunosorbent assay
FcRγ	Fc receptor gamma
FRET	Fluorescence resonance energy transfer
GAG	Glycosaminoglycans
GPCR	G-protein coupled receptor
GPIbα	Glycoprotein Ib, von Willebrand Receptor
GPVI	Glycoprotein VI
HEK cells	Human embryonic kidney cells
HUVEC	Human umbilical vein endothelial cells
ITAM	Immunoreceptor tyrosine-based activation motif
KRG	Krebs-ringer glucose buffer
LAMP3	Lysosome-associated membrane glycoprotein 3
LAT	Linker for Activation of T cells
LPS	Lipopolysaccharide
MAP-tool	Molecule activity prediction tool
MYPT1	Myosin phosphatase target subunit 1
Ni-NTA	Nickel-nitrilotriacetic acid
NO	Nitric oxide
P2X1	Purinergic receptor 2X1, ATP binding ligand gated ion channel

P2Y1	Purinergic receptor 2Y1, receptor for ADP and ATP
P2Y12	Purinergic receptor 2Y12, receptor for ADP
PAC-1	Procaspase activating compound 1
PAR	Protease activated receptor
PEA	Proximity extension assay
PEAR1	Platelet endothelial aggregation receptor 1
PI3K	Phosphoinositide 3-kinase
PLCγ2	Phospholipase C gamma 2
PVDF	Polyvinylidene difluoride
qPCR	Quantitative real-time polymerase chain reaction
RAFT	Reversible addition-fragmentation chain-transfer
RNA	Ribonucleic acid
ROCK	Rho-associated protein kinase
S.E.M	Standard error of mean
SFK	Src family kinases
SFLLRN	Single amino acid code for the thrombin activating peptide
SNAP	S-Nitroso-N-acetyl-DL-penicillamine, NO-donor
Src	Proto-oncogene tyrosine-protein kinase Src
Syk	Spleen tyrosine kinase
TNF-α	Tumour necrosis factor alpha
TPα	Thromboxane A2 receptor alpha
VASP	Vasodilator-stimulated phosphoprotein

Introduction

This chapter will first address the normal physiology of platelets, after which pathological consequences of dysfunctional haemostasis will be addressed. This introduction will describe the molecular mechanisms underlying platelet activation and provide an overview to the background of the thesis.

Platelets in haemostasis

Platelets are small cytoplasmic fragments derived from megakaryocytes within the bone marrow. Platelets are guardians of maintained haemostasis and key players in pathological arterial blood clot formation, i.e. thrombosis. The initiation of primary haemostasis and subsequent platelet aggregate formation is a complex interplay of activating and inhibiting signalling processes. The blood vessel endothelial wall releases platelet inhibitory substances such as nitric oxide (NO) and prostaglandin I2. In case of vessel wall injury or rupture of atherosclerotic plaque, platelets will be exposed to the subendothelial matrix. The subendothelial matrix contains molecules like collagen and von Willebrand factor. These molecules cause platelet activation via surface glycoproteins, and various cell adhesion molecules such as integrins. Platelet activation leads to shape change, adhesion, release of granules and lysosomes, aggregation, and formation of a pro-coagulant surface. Upon activation, platelets generate the eicosanoid thromboxane A2, which functions as an autocrine and paracrine enhancer of platelet aggregation. Furthermore, coagulation will reinforce platelet activation by generated thrombin acting on protease-activated receptors (PARs) [1,2].

Platelets contain two types of granules; dense granules and alpha granules. Dense granules contain adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin and calcium [2]. Proteomics analysis of granule content has reviled that alpha granules may contain hundreds of different proteins that are involved in a multitude of different physiological functions [3,4].

Atherosclerosis and thrombosis

According to the World Health Organization, cardiovascular diseases are the leading cause of death worldwide [5]. The main underlying cause of cardiovascular disease is atherosclerosis. The pathogenesis of atherosclerosis and its acute clinical complications like myocardial infarction comprises endothelial dysfunction, inflammation, and intimal lipoprotein accumulation in the vessel wall. A complex inflammatory process finally results in risk for plaque rupture and pathological calcification. Platelets are central in thrombus formation.

Plaque erosion or rupture cause misdirected platelet activation, thrombus formation, and possible blood vessel occlusion. Blood vessel occlusion gives rise to ischemic damage and tissue death in vital organs such as the heart and brain [6].

Anti-platelet drug therapies

The standard treatment strategies to prevent re-occurrence of myocardial infarction and stroke consists of drugs that inhibit coagulation or platelets. The most used treatment against platelet activation involves inhibition of the aggregation enhancing feedback loops, specifically the ADP receptor P2Y12 [7,8], or irreversible inhibition of cyclooxygenase 1 (COX1) by aspirin, leading to supressed thromboxane A2 generation [2,9,10]. However, irreversible inhibition of COX1 or platelets can have adverse effects such as severe gastrointestinal bleeding, especially during trauma or when unplanned surgery is required [9,11]. Haemostasis is a balance act between severe bleeding and unwanted thrombus formation that is in dire need of new treatment approaches and drugs to achieve beneficial effects with reduced risk of adverse events.

Molecular mechanisms of platelet activation and inhibition

The platelet surface contains a large number of receptor proteins with both activating and inhibiting properties. Some of the most studied surface receptors on platelets belong to the family of G-protein coupled receptors (GPCRs). Platelets express multiple G-protein alpha subunits, including $G_{\alpha q}$, $G_{\alpha 13}$, $G_{\alpha s}$ and $G_{\alpha i}$ [1]. The $G_{\alpha q}/G_{\alpha 13}$ coupled PAR receptor 1 and 4 are powerful platelet activating receptors that recognise thrombin. Purinergic receptor P2Y1 is $G_{\alpha q}$ coupled while P2Y12 couples to $G_{\alpha i}$. Other activating GPCRs include the thromboxane A2 receptor (TPα) which couples to $G_{\alpha q}$ and $G_{\alpha 13}$. However, receptor coupling to $G_{\alpha s}$, such as the prostacyclin receptors has been described to inhibit platelet activation through elevation of cyclic adenosine monophosphate levels (cAMP) [12].

One of the most abundantly expressed receptors on the platelet surface is the fibrinogen recognising integrin receptor $\alpha_{IIb}\beta_3$ expressing up to 80 000 copies per cell [1]. Platelets express multiple integrin receptors, supporting adhesion to the subendothelial matrix. Collagen exposure is an important part of platelet adhesion and activation to the subendothelial matrix, involving activation of the collagen receptor Glycoprotein VI (GPVI). Adhesion is

supported by von Willebrand factor binding of the GPIb-V-IX receptor complex on the platelet surface [13]. Platelets express several Immunoreceptor Tyrosine-Based Activation Motif (ITAM)-containing [1] receptors, including collagen recognising receptor GPVI which signals together with FcRγ [14]. Another ITAM receptor on platelets is C-type Lectin-2 (CLEC-2) that recognises podoplanin, a protein expressed in endocrine vessels [15]. Signalling through ITAM receptors takes place via the downstream Src family kinases, spleen tyrosine kinase (Syk), Linker for Activation of T cells (LAT) and Phospholipase C gamma 2 (PLCγ2) [16–18].

The role of platelets expands beyond haemostasis

The field of platelets in inflammation and innate immunity has emerged in recent years. The platelet surface contains various immune receptors including nine types of toll-like receptors [13,19]. Platelets have been shown to interact directly with immune cells and cause immune cell activation through release of granule content [20]. In addition, platelets can bind directly to pathogens. Platelets can activate the vascular endothelium giving rise to increased endothelial permeability, facilitating for leukocyte infiltration. Direct interaction with immune cells and endothelial cells is mediated by P-selectin (CD62P), which is exposed on the platelet surface after activation. In addition, platelets release other immune related and regulatory substances during activation [13].

Through proteomic analysis, over 4000 platelet proteins have been identified, some of which the function still is undescribed [21]. Similarly, some surface receptors still have no identified ligand, or the function of the receptor is yet to be explored [22]. The precise roles of platelets in innate immunity are not fully understood and are a subject of intensive research.

Carbohydrates as modulators of haemostasis

Glycans participate in a large number of biological processes, including cell-cell adhesion, self-recognition and other immune responses. This broad group of biomolecules has profound effects on haemostasis [23]. One of the most studied modulators of haemostasis is heparin. This sulfated glycosaminoglycan was introduced commercially in the 1920s. Heparin comprises a mixture of polysaccharides, and is closely related to dermatan sulfate, hyaluronic acid, and heparan sulfate. The effect of heparin on the coagulation cascade mainly takes place via inhibition of thrombin (coagulation factor IIa)

[24]. Heparin binds to anti-thrombin via a specific penta-saccharide sequence [25]. Heparin has been shown to interact with GPIbα, a component in the GPIb-V-IX receptor complex, inhibiting thrombin induced platelet activation [26]. Administration of heparin can lead to direct platelet-heparin interactions which can cause fatal adverse effects such as heparin-induced thrombocytopenia, a pro-thrombotic condition [27].

Heparins and heparan sulfate are not the only carbohydrates able to affect primary haemostasis. A heparin-like sulfated polysaccharide with glucoside backbone, dextran sulfate, has been shown to induce platelet aggregation [28] via the platelet endothelial aggregation receptor 1 (PEAR1) and CLEC-2 [29]. Sulfated fucose polysaccharides from marine seaweed has been shown to interact with platelet P-selectin [30], and have the ability to activate platelets via CLEC-2 [18]. Figure 1 illustrates the signalling pathways of CLEC-2 and PEAR1 with their currently known agonists in comparison to the well-characterised collagen receptor GPVI, which displays a similar intracellular signalling pattern to CLEC-2.

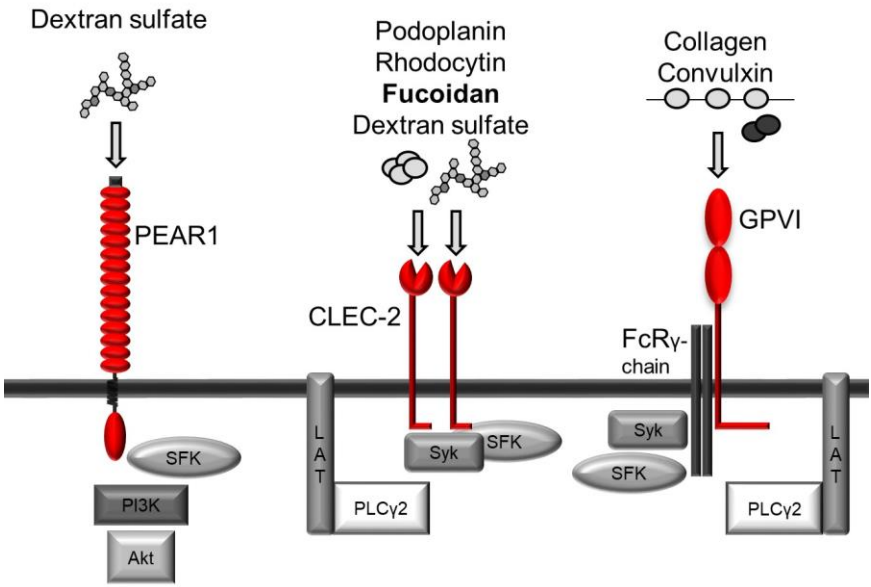

Figure 1 Illustration of PEAR1, CLEC-2 and GPVI-signalling with their known downstream signalling molecules as described prior to this thesis..
Created by Caroline Kardeby.

Synthetic glycopolymers and natural fucoidans

Natural sulfated fucoidans are fucose-rich polysaccharides, derived from brown seaweed and echinoderms. Natural fucoidans display a wide range of biological functions, they have been described to have anti-microbial, anti-inflammatory and anti-cancer properties [31]. In addition, natural fucoidan is a regulator of haemostasis. Dependent on their origin, fucoidans have different effects on both primary haemostasis [32] and coagulation. Both pro-coagulant [33,34] and anti-coagulant [32,35] effects have been reported. Some discrepancies between the observed effects may be attributed to the varying chain length, branching, and degree of sulfation [32,36–38]. Extraction method and even parameters such as season of harvest can affect the end product [39].

The mechanism of action which could explain the biological function of fucoidan is difficult to study due to the heterogenous nature of marine carbohydrates. For this reason, the ability to synthesise sulfated α -L-fucoside pendant glycopolymers with fucoidan-mimetic properties, gave rise to a unique opportunity to study the mechanism of sulfated fucose polysaccharides. By precise and novel synthesis processes variables such as structural variation was removed, and degree of sulfation was more homogenous [40].

Scientific background to the start-up of the thesis

In 2012 an integrative research project was initiated between Örebro University and Linköping University in order to synthesise sulfated α -L-fucoside pendant glycopolymers with potential anti-viral, anti-inflammatory and anti-coagulative properties. It has been shown that bacteria and viruses are equipped with heparan sulfate binding proteins that can help them to attach to host cells and facilitate their ability to enter the cells [41]. Studies have shown that exogenously added heparin and heparan sulfate can inhibit spreading of viruses such as herpes simplex virus and human papillomavirus [41]. The synthetic sulfated glycopolymers with fucoside backbone were successfully able to decrease viral spreading by HSV-1. However, they showed platelet activating properties, in similarity to natural fucoidan [40].

In 2012 another synthetic construct was produced by collaborators at the National Hellenic Research Foundation in Athens, Greece [42]. The purine analogues made by the Greek research group had been described as anti-inflammatory and potential NO-releasing compounds [43]. Early pilot studies from our laboratory revealed that the purine analogues inhibit platelet activation.

Both regarding synthetic sulfated glycopolymers and synthetic purine analogues, the mechanism underlying modulation of platelet functions were unknown.

Aims

The aim of the thesis was to study platelet signalling and endothelial cell responses using unique synthetic drugs. Through providing new molecular insights in how synthetic molecules with therapeutic potential interact with cellular targets the results of this thesis will provide new knowledge that can be used for the development of future anti-thrombotic or anti-inflammatory drugs.

The specific aim of each paper was as follows:

I. To investigate the effect of synthetic glycopolymers on platelets and determine the role of chain-length and sulfation.

II. To establish the molecular mechanisms underlying platelet activation by synthetic glycopolymers and natural polysaccharides.

III. To investigate the effect of synthetic glycopolymers and natural polysaccharides on endothelial cells, with a focus on inflammation related proteins.

IV. To characterise the effect of a synthetic purine analogue with a nitrate ester motif on platelet aggregation and secretion and determine the underlying molecular mechanism.

Methodology

This section will describe the biological material, synthetic constructs, and methods performed in the different papers of the thesis.

Biological material

Paper I, **II** and **IV** use human blood samples. **Paper II** uses mouse blood samples. **Paper III** only uses material from cell culture experiments.

Human blood sampling

Blood samples were collected from self-reported healthy volunteers which had not consumed non-steroid anti-inflammatory drugs like aspirin during the past two weeks. Blood samples were collected in heparinised tubes by venepuncture.

Isolation of human platelets

Blood platelets were isolated through a two-step centrifugation protocol. Blood samples were mixed in a ratio 1:5 with a citric-acid-dextrose (ACD, 71 mM citric acid, 85 mM sodium citrate, 111 mM glucose) solution. In the first centrifugation step, at 220 × g for 22 min, the blood will separate in three visible phases, the platelet-rich plasma, the buffy coat containing white blood cells, and a layer of red blood cells in the bottom half of the tube. Platelet-rich plasma was carefully collected, leaving approximately a half centimetre of plasma above the buffy-coat to avoid high levels of white blood cell contamination in the final platelet suspension. The platelet-rich plasma was supplemented with additional ACD at a ratio of 1:10 before the second centrifugation step. For calcium measurements in **Paper II** and all methods in **Paper IV** the platelet-rich plasma was supplemented with 1 U/ml apyrase to minimise autocrine-paracrine P2-receptor desensitisation by unwanted ADP release during centrifugation. After the second centrifugation step, at 480 × g for 17 min, the platelets were collected at the bottom of the tube in the form of a pellet. The pellet was gently washed three times using Krebs-Ringer Glucose buffer (KRG) without calcium chloride, supplemented with 0.05 U/ml apyrase. The pellet was carefully resuspended to 2.5×10^8 platelets/ml in KRG with 0.05 U/ml apyrase, supplemented with 1mM $CaCl_2$.

Mouse models

Paper II uses blood samples from three different mouse models which are briefly described below.

Pear1$^{-/-}$

Experiments using Pear1$^{-/-}$ mice were performed in KU Leuven, Belgium, by our collaborator doctor Maarten Criel in professor Marc F. Hoylaerts' laboratory. In brief, Pear1$^{-/-}$ mice were obtained by breeding Pear1+/- mice (Pear1$^{tm1a(KOMPW)tsi}$; C57BL/6N-background), the mice were genotyped to confirm the absence of the Pear1 gene [44]. It has previously been shown that the sulfated polysaccharide dextran sulfate induces aggregation that depends both on PEAR1 and CLEC-2 [29]. Hence, in **Paper II** the Pear1$^{-/-}$ mice were used to investigate the necessity of the PEAR1 receptor in mouse platelet aggregation to synthetic glycopolymers.

Clec1b$^{fl/fl;PF4\text{-}Cre}$

Experiments using blood Clec1b$^{fl/fl;PF4\text{-}Cre}$ mice were performed at University of Birmingham by me and our collaborators doctor Elisabeth J. Haining and Stephanie Watson in professor Steve P. Watson's laboratory. The complete absence of *Clec1b* in mice is lethal in most offspring [45]. Hence a conditional knockout was used. The Clec1b$^{fl/fl;PF4\text{-}Cre}$ mouse used in this study lack *Clec1b* in cells expressing *Pf-4*, limiting the knockout effect to involve megakaryocytes and platelets [45,46]. Previously it has been shown that natural fucoidan is unable to cause aggregation of platelets from Clec1b$^{fl/fl;PF4\text{-}Cre}$ mice [47]. In **Paper II** the Clec1b$^{fl/fl;PF4\text{-}Cre}$ mice were used to investigate if the synthetic glycopolymers behaved the in the same way as natural fucoidan.

hIL-4Rα/GPIbα transgenic mice

Experiments using the hIL-4Rα/GPIbα-transgenic mice were performed in professor Steve P. Watson's laboratory. In the hIL-4Rα/GPIbα-transgenic mice the extracellular domain of GPIbα is replaced to human IL-4 receptor α [48]. In **Paper II** the hIL-4Rα/GPIbα-transgenic mice were used to investigate how mouse platelet aggregation to synthetic glycopolymers was affected by the lack of the extracellular domain of GPIbα.

Isolation of mouse platelets

Blood samples from mice was collected by staff with special training in mouse handling. Blood was collected in a syringe with ACD from vena cava in CO_2 terminally anaesthetised mice. The mouse-platelet isolation was performed using centrifugation, similarly to the human platelet isolation protocol. The blood was centrifuged at 200 × g for 6 min to obtain platelet-rich plasma. Second step centrifugation was at first performed at 1000 x g for 6 min in the presence of 0.1 μg/ml prostacyclin. However, we noticed that the platelet preparations using 1000 × g yielded low platelet aggregation amplitude using synthetic glycopolymers, hence we modified the protocol to use 500 × g for 9 min instead. The lower g-force doubled the aggregation amplitude to synthetic glycopolymers. In **Paper II** we decided to exclusively use the protocol with lower g-force. Mouse platelets were used at a density of 2 × 10^8 /ml.

Cell culturing

Human umbilical vein endothelial cells (HUVEC)

In **Paper III**, pooled human umbilical vein endothelial cells (HUVECs) were cultured in T-75 flasks in complete Vasculife® medium. The cells were maintained at 37°C in 5% CO_2. Upon confluency the cells were detached and seeded for experiments. The stimulations using synthetic glycopolymers and natural polysaccharides were performed in the absence of antibiotics. Cells were seeded at a density of 6 × 10^4 in 24-well plates or 3 × 10^5 in 6-well plates. Stimulations were carried out for 1h, 4h, 12h, 24h, and 48h. Cells were used in passage 4 to 9.

Human embryonic kidney 293 cells (HEK 293)

In **Paper II** human embryonic kidney 293 cells (HEK 293) were cultured for the purpose of expressing recombinant proteins. Cultures of HEK 293 cells were maintained in Freestyle 293 medium, supplemented with 10% kollipher and 0.05% G418 in Erlenmeyer cell culture flasks. Cells were cultured under agitation at 37 °C in 5% CO_2.

Subcloning

In **Paper II** synthetic biotinylated glycopolymers were tested for binding against full length PEAR1, EGF-like repeat 1-12, 13 alone, and 12-14. Plasmids of full length PEAR1 and truncated fragments were designed by doctor Yi Sun from the University of Birmingham [49]. Plasmids were diluted to 10 ng/ml and combined with a suspension of α-select bronze efficiency bacteria, a type of *Escherichia coli* used for subcloning. The plasmids were heat-shocked into the bacteria through incubating the bacteria in a water bath of 42°C for 50 seconds before they were returned to 4°C. The bacteria were incubated for 30 min at 37°C before they were spread on agar ampicillin plates and incubated overnight. Bacteria that successfully express the plasmid will express a gene for ampicillin resistance as well. Using antibiotics-supplemented medium promoted survival of plasmid-bearing bacteria. The following day a single large colony from the ampicillin plate was transferred to a flask of lysogeny broth supplemented with ampicillin to incubate overnight once more. On the third day the plasmids were isolated using a commercial maxiprep kit.

Transfection

In **Paper II** recombinant proteins and protein-fragments were expressed in HEK 293 cells through transient transfection. The day before transfection HEK 293 cells were seeded into new flasks using 1.25×10^7 cells per flask. Freestyle 293 medium without supplements was used for transfection. On the day of transfection, medium was combined with plasmid solution and polyethyleneimine. The transfection solution was allowed to pre-complex for 5 minutes before it was added to the cell culture flasks. The cell culture flasks were returned to the incubator and left for six days before protein harvest. On the day of harvest all content of the cell culture flask was centrifuged in falcon tubes. Proteins were purified from the medium using their His-tag using Ni-NTA Resin.

Organic chemistry and synthesis

Synthesis of sulfated glycopolymers

In **Paper I**, **II**, and **III** synthetic glycopolymers with fucoidan-mimetic properties were used. The glycopolymer synthesis was performed by doctor Mattias Tengdelius in professor Peter Konradsson's laboratory at Linköping University, Sweden. The glycopolymers were synthesised using two different methods. The longer polymer was created using thiol-mediated free radical chain transfer polymerisation [50] and the shorter polymers were synthesised using reversible addition-fragmentation chain-transfer (RAFT) polymerisation to achieve a better control over glycopolymers chain-length (**Paper I**) [36]. In **Paper I** the glycopolymers nomenclature was based on the order in which the glycopolymers were synthesised. However, in **Paper II** and **III** we choose to rename the glycopolymers to names based on the average monomeric chain-length. The glycopolymer nomenclature used in **Paper I**, **II** and **III** is clarified in Table 1. For the sake of simplicity, the names based on average monomeric chain-length will be used from this point onward.

*Table 1 Clarification of changes in nomenclature of synthetic glycopolymers between **Paper I** and **Paper II** to **III**.*

Synthesis method	Paper I	Paper II and III
Thiol-mediated free radical chain transfer	Glycopolymer 2*	NSC329*
	Glycopolymer 3**	C329**
Reversible addition-fragmentation chain-transfer	Glycopolymer 7**	Not used
	Glycopolymer 9**	C13**

* Non-sulfated glycopolymer

** Sulfated glycopolymer

Both the synthesis methods applied to create the glycopolymers are radical polymerisation techniques. Radical polymerisation can be defined as a process where monomeric units are linked together into chains where radicals function as kinetic-chain carriers [51].

All glycopolymers were created using fucoside monomers (2-methacrylamidoethyl 2,3,4-tri-O-acetyl-a-L-fucopyranoside), in both synthesis methods the glycopolymers length is in part regulated by the ratio between monomeric units and chain-transfer agent (CTA). Radical polymerisation can be divided into three steps; initiation, propagation, and termination.

The initiation of polymerisation takes place when a radical is donated by the initiator. In both synthesis methods AIBN (2,2′-Azobis(2-methylpropionitrile)) was used as initiator. Upon exposure to heat AIBN will degrade, releasing a radical which initiates propagation.

Propagation is the step in which the polymer chains will increase in length. When the free radical reacts with methacrylamid group of the monomeric unit it will change the double-bound carbon in to more stable carbon-carbon bond, pushing a new free electron to be available for interaction with another monomeric unit, resulting in chain growth.

Termination occurs when the polymer chain loses its free radical. There are multiple ways in which the loss of radical can occur some examples are; A hydrogen transfer between the polymer chains, or through the radical being replaced by a hydrogen atom. Termination can occur at random, and the risk increases the fewer monomeric units that are available for propagation.

One of the most significant differences between the two synthesis methods is in the way the termination occurs, which is the reason for increased control achieved when using RAFT. In thiol-mediated chain transfer polymerisation 2-(trimethylsilyl)- ethanethiol is used as a CTA, which controls the polymerisation through termination of one polymer chain, while initiating the next. This process is partly dependent on the CTA-monomer ratio, and partly random, giving some control of the termination step and the chain length of the glycopolymers. On the other hand, the CTA used in RAFT, known as RAFT agent, was CPBDT (2-cyano-2-propyl benzodithioate). The unique feature of RAFT agents is that they can pause the polymer chain propagation without terminating it, putting the chain in a state called equilibrium. Through a complex chemical interaction, the RAFT agent will pause and unpause propagating chains instead of terminating them, giving rise to an even growth of the polymer chains and thus resulting in a more controlled polymer chain growth, and lower polydispersity.

After polymerisation, the glycopolymers were O-sulfated by treatment with sulfur trioxide pyridine complex. The sulfation degree of the constructs was 86-89% [36,50], similarly to natural fucoidan [31].

Chemical structure of purine analogues

In **Paper IV**, we utilised synthetic compound 6-[4-(6-nitroxyacetyl)piperazin-1-yl]-9H-purine, denoted MK128 (Figure 2). The purine analogue MK128 was created by doctor Maria Koufaki and doctor Theano Fotopoulou at the National Hellenic Research Foundation, Institute of Biology, Medicinal Chemistry & Biotechnology, in Athens, Greece. The synthesis of MK128 (known as compound 15) was described in full detail 2012 by Koufaki et al. [42]. MK128 consists of three potential pharmacological active structures: a purine and a piperazine motif as well as a nitrate ester motif in the end of the side chain (Figure 2). In earlier studies by doctor Koufaki, beneficial effects of MK128 has been attributable to the nitrate ester motif [52].

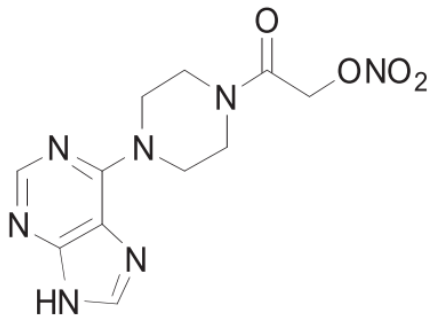

Figure 2 Chemical structure of MK128.

Molecular and functional methods

Light transmission aggregometry and secretion

Platelet aggregation is a biological function which is essential for maintaining primary haemostasis. Light transmission aggregometry is a method for measuring the ability of platelets to aggregate *ex-vivo*. Briefly, aliquots of platelet suspension are added to silicone coated glass cuvettes containing a small metal stir bar. The cuvettes are placed in a temperature-controlled machine containing a magnetic stirrer (Chronolog 700 model). The light transmitted through the cuvette is measured and calibrated against another cuvette containing the platelet medium alone, in this case KRG. The blank cuvette represented 100% light transmission, corresponding to maximal platelet aggregation. The level of light transmission of the resting platelet sample will be set as a baseline, representing 0%. As platelets get stimulated with agonists they aggregate, and this will result in an increased light transmission. The results can be quantified as a percentage increase in light transmission. **Paper I**, **II**, and **IV** use aggregometry to investigate the effect of synthetic glycopolymers or purine analogue MK128 in the presence or absence of various agonists and inhibitors. Light transmission was measured at 37 ^{o}C, at a stirring speed of 900 rpm. The Chronolog Lumi-aggregometer used in **Paper I**, **II** and **VI** can simultaneously measure chemiluminescence. By using a luciferin/luciferase substrate it is possible to measure secretion simultaneously with aggregation. The release of ATP can be quantitively estimated using a standard of known ATP concentration. Secretion of ATP was measured in **Paper I** and **IV**.

Intracellular Ca^{2+} measurements

When activation occurs a rapid rise in cytosolic calcium will take place. Measurements of cytosolic calcium mobilisation can be a useful and robust method in studies of platelet signalling. **Paper II** and **IV** use Fura-2 technique to register increases of cytosolic calcium. Fura-2 AM is a fluorescent probe conjugated with acetoxymethyl ester which allows passive diffusion through the platelet membrane. When Fura-2-AM enters the platelet the acetoxymethyl ester will be cleaved off by esterase, making the Fura-2 unable to leave the cell. Fura-2 is excited at two different wavelengths, 340 and 380 nm. When the probe is bound to calcium its fluorescent properties at 510 nm is changed. In **Paper II** and IV the increase in cytosolic calcium is illustrated as increase in fluorescence ratio 340/380 nm.

Flow cytometry

Flow cytometry is a method that uses laser technology to register fluorescence intensities of single cells. The expression of proteins can be detected using fluorescence labelled antibodies. Upon platelet activation, surface expressions of various proteins will change. Alpha granules and dense granules will fuse with the platelet membrane, leading to exposure of granule membrane proteins on the platelet surface. In addition, surface receptors may change their conformation. Well-known surface markers of platelet activation are P-selectin from alpha granules, LAMP3 from dense granules and lysosomes, and the conformational change of integrin $\alpha_{IIb}\beta_3$. In **Paper I** the changes in platelet surface expression caused by synthetic glycopolymers and natural fucoidan was investigated.

Since the measurements were performed in whole blood, the platelet marker CD42b (GPIbα) was included in the analysis to facilitate platelet detection. In short, whole blood was combined in tubes with buffer, antibodies, and agonists. After 10 minutes the samples were diluted using additional buffer to slow down further activation or platelet autocrine-paracrine stimulation. Finally, samples were analysed using the Gallios flow cytometer from Beckman Coulter. Samples were compared to untreated controls for increases in protein surface expressions of P-selectin, LAMP3, and binding of the PAC-1 antibody, a marker for integrin αIIbβ3 activation.

Most cells and solutions give rise to some form of background fluorescence, and certain surface expressions may be weak, especially when lower concentrations of agonists are used. To determine the limit between positive and negative cells isotype controls are used. Isotype controls are non-specific antibodies with the same fluorophore as its specific counterpart. The isotype controls were used to set the gates, to determine the difference between positive and negative. Data was presented as percentage positive platelets. Treated samples were compared to untreated controls.

Immunoprecipitation

In **Paper II** immunoprecipitation was used to study phosphorylation of PEAR1. To date, there are no specific PEAR1 phospho-antibodies. Immunoprecipitation is a method that can be used to single out proteins from complex biological samples. Aliquots of platelet suspension were stimulated using synthetic glycopolymers and natural polysaccharides. After stimulation, the platelet samples were lysed and incubated with PEAR1 specific antibodies overnight to allow antibody-antigen binding. Gel beads able to capture the antibodies were used to clear the lysate from the antibodies. The beads were washed in several steps after which the bead content was eluted and subsequently analysed by western blot.

Western Blot

In **Paper I**, **II** and **IV** western blots were applied to study phosphorylation of platelet proteins with a potential role in the signalling cascades affected by the synthetic drugs. Western blot is a method in which relative protein quantities and phosphorylation states can be investigated. Aliquots of platelet suspensions were treated with various agonists and inhibitors for specific time periods and lysed using a sodium-dodecyle sulfate sample buffer. After lysis, the samples are heated to promote denaturation. In the denaturation process the proteins will be negatively charged by the lysis buffer, making them able to travel through the gel in the direction of the electric current. The denatured protein solutions were loaded onto gels using the same loading volume for all samples. Alongside the samples a protein marker was added. A protein marker contains proteins of specified sizes and works as a support in determining the protein size of proteins of interest. The western blot gels used in **Paper I**, **II** and **IV** are gradient gels with increasing density of polyacrylamide. Proteins will be separated by electrophoresis according to size. After protein separation the proteins within the gel will be transferred on to a polyvinylidene difluoride (PVDF) membrane. After protein transfer the membrane will be blocked for unspecific interactions using bovine serum albumin in tris-buffered saline with tween and subsequently probed using antibodies. In **Paper I**, **II**, and **IV** both antibodies against specific phospho-sites as well as antibodies for total protein were used. In study I and II pan-phospho-tyrosine antibody 4G10 was used. 4G10 will stain all tyrosine phosphorylated protein residues in the samples. Using 4G10 it is possible to investigate phosphorylation patterns on an overview scale, or to analyse the phosphorylation status of immunoprecipitated proteins.

Enzyme linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is commonly applied to measure protein quantities. In **Paper III** a commercial sandwich ELISA is used. A classical sandwich ELISA incorporates two antigen-specific antibodies. A capture antibody will be incubated in a 96-well plate, after which the biological material will be added, in **Paper III** cell culture medium was used. After incubation with the sample a second capture antibody will be added to the plate and the protein of interest will be sandwiched between two different antigen-specific antibodies. In this specific assay the detection antibody is coupled with biotin, which will interact with streptavidin-conjugated horseradish peroxidase. Upon the addition of substrate, the horseradish peroxidase will catalyse the oxidation of the substrate causing a colorimetric change which can be detected using a spectrophotometric plate reader. The detected amount of protein can be relatively quantified through calculation, comparing the change in optical density of the sample to a standard curve.

Olink® Proximity Extension Assay (PEA)

In **Paper III** samples are sent for analysis with the company Olink® Proteomics. HUVECs were treated with synthetic glycopolymers and natural polysaccharides for 24h and 48h. Cell culture medium from 24h and 48h was analysed using Inflammatory panel from Olink®, while cell lysates from 48h was analysed using the Cardiovascular III panel. Each Olink® panel analyses 92 different biomarkers using a proximity extension assay (PEA) technology. In brief, two antigen-specific nucleotide-labelled matched antibodies will bind to the protein pairwise. When the two nucleotide sequences are in the proximity of each other it facilitates for DNA-amplification of the nucleotide sequences. The amplified sequence will be measured using real-time polymerase chain reaction (qPCR). The data generated by Olink® Proteomics is reported back as Normalised Protein eXpression (NPX) values, which are relatively quantified values in $\log^2$ scale.

Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Quantitative Real-Time Polymerase Chain Reaction (qPCR) is a commonly used method to study gene expression. In **Paper III** RNA was isolated from cell lysates, after which cDNA synthesis was performed. The cDNA was combined with TaqMan primers and probes specifically designed to for the gene of interest. In the PCR-mixture, DNA polymerase was added, deoxy nucleoside triphosphates (dNTPs), and a buffer containing magnesium and potassium ions.

Through thermocycling the DNA strands will be amplified. In brief, DNA strands get melted into single strands, after which the primers will attach to the single strands and the DNA polymerase will initiate elongation. Fluorescence labelled TaqMan probes were used. TaqMan probes utilise Fluorescence Resonance Energy Transfer (FRET) for detection. During amplification, the probe will hybridise with the DNA. When the *Taq* polymerase encounters the probe, the probe will be degraded. The degradation of the probe leads to separation of the fluorophore and the quencher, giving rise to an increased fluorescence for each newly created amplicon.

The analysis included a negative control that consisted of RNAse free Milli-Q water instead of DNA which was used for monitoring DNA-contamination of reagents. To control for possible variations in cell count, all results were normalised against a housekeeping gene. In **Paper III** glyceraldehyde 3-phosphoate dehydrogenase (GAPDH) was used as a house-keeping gene. All expression values were calculated from a pooled 6-point standard curve incorporated in the analysis and normalised to GAPDH.

Molecular interaction methods

AVidity-based Extracellular protein Interaction Screen (AVEXIS)

In **Paper II** glycopolymer-protein interactions were investigated using Avidity-based Extracellular protein Interaction Screen (AVEXIS). In brief, streptavidin coated plates were incubated with biotin labelled sulfated and non-sulfated glycopolymers for 1h after which the platelets were washed, and β-lactamase tagged proteins and protein fragments expressed in HEK 239-cells through transfection were added. After 1h of incubation the plates were washed again and incubated for 1h with 125 μg/ml nitrocefin. Absorbance was measured at 485 nm. As nitrocefin is degraded by β-lactamase it will shift in colour and turn from yellow to red. Hence, proteins bound to the glycopolymers will induce a colour shift which can be measured by absorbance. Prior to AVEXIS the proteins were normalised for β-lactamase activity as previously described [53].

Kinase Activity Fluorescence Resonance Energy Transfer (FRET) Assay

In **Paper IV** the novel purine analogue MK128 was evaluated as a potential kinase inhibitor using the SelectScreen™ Kinase Profiling Services. A list of 18 kinases out of interest for platelet signalling were selected for screening. All 18 kinases were tested against 30 μM MK128. Two different technologies were used to evaluate kinase activity; Z'-LYTE and Adapta®. Both technologies are based on FRET. In short, FRET is a fluorescence-based technique in which the energy released upon excitation of a donor molecule can be transferred to an acceptor molecule if it is in the nearby vicinity.

The Z'-LYTE technology detects changes in kinase-induced phosphorylation of substrates. Z'-LYTE uses substrates labelled with coumarin as a donor, and fluorescein as an acceptor. If the donor and the acceptor are near each other the donor signal will be lower since energy will be transferred to the acceptor molecule. If the kinase becomes unable to phosphorylate the substrate, the substrate will be cleaved by a development reagent which will cause the cleaved substrates to emit fluorescence mainly in the donor's wave-length since energy is no longer transferred to the acceptor.

The Adapta® assay detects ADP formation using FRET. The kinase will convert ATP to ADP. Subsequently, europium-labelled anti-ADP antibodies and Alexa Flour®647 labelled ADP-tracers are added to the mixture. The ADP-antibody will bind to successfully formed ADP instead of binding the tracer giving a low FRET signal. However, if the kinase is inhibited and ATP remains in the solution, the ADP antibody will bind to the ADP tracer instead

giving rise to a high FRET signal and increased fluorescence of the acceptor molecule (Alexa Fluor®647).

The fluorescence signal registered will be converted using mathematical formulas and SelectScreen™ will report a percentage of inhibition per kinase.

Statistical analysis

All four Papers present data as a mean and standard error of mean (S.E.M). Statistical tests were carried out using GraphPad Prism 6. Statistical compar-ison between two groups was performed using student's T-test. Upon com-parison of multiple groups one-way analysis of variance (One-way ANOVA) with Bonferroni's post-hoc test was performed. In **Paper III** p-values were corrected for multiple testing using Benjamini-Hochberg's method in the software "R", since this function was not available in GraphPad Prism.

Ingenuity Pathway Analysis

In **Paper III** the protein data is analysed using Ingenuity® Pathway Analysis and compared against the Ingenuity Knowledge Base, a database maintained by QIAGEN Bioinformatics. The database is updated four times per year with new molecular relationships from peer-reviewed papers. The data analysis of **Paper III** was performed at 2018-12-15, right after a database update. **Paper III** contains results from "Upstream Regulator Analysis", which uses Fisher's Exact T-test to calculate the p-value of overlap. In **Paper III** the Molecule Activity Prediction (MAP) tool is used to estimate the direction of activity for selected functions in customised networks. The MAP-tool uses a Z-test to estimate function activity.

Ethical consideration

This thesis abides the Helsinki Declaration. This research involves the use of human platelets from blood of healthy volunteers. Blood sampling was approved by the Regional Ethical Review Board in Uppsala, Sweden (Dnr 2015/543). All procedures involving murine blood were performed follow-ing United Kingdom Home Office approval (PPL P0E98D513) or approved by the local Ethics Committee of the KU Leuven in Belgium.

Results and discussion

The overall aim of this thesis was to study platelet signalling and endothelial cell responses using unique synthetic drugs. The thesis provides new molecular insights in how synthetic molecules interact with cellular targets. This section briefly describes and discusses the key results from **all four Papers** in relation to the aims of the thesis. Each paper provides a more detailed description of all results.

Sulfated synthetic glycopolymers activate platelets

In **Paper I** the aim was to characterise the effect of synthetic sulfated α-L-fucoside pendant glycopolymers on platelets. In **Paper I**, sulfated glycopolymers of three lengths were compared to non-sulfated glycopolymers and natural fucoidan. Interestingly, all three chain-lengths of synthetic sulfated glycopolymers caused aggregation and secretion in isolated platelets, to a similar amplitude of natural fucoidan. However, non-sulfated glycopolymers did not cause any platelet aggregation. The observed aggregation caused by sulfated glycopolymers requires integrin αIIbβ3 activation, indicating for an active signalling process rather than GPIbα mediated agglutination. This was confirmed by studying the intracellular tyrosine phosphorylation pattern over time using western blot. Synthetic sulfated glycopolymers of three different lengths cause intracellular phosphorylation patterns. By using western blot analysis, we observed a rapid and prominent tyrosine phosphorylation occurring at 125 kDa, which later turned out to be of major importance.

Using flow cytometry on whole blood, we showed that synthetic glycopolymers and natural fucoidan from *Fucus vesiculosus* cause a dose-dependent increase in platelet surface expressions of P-selectin, LAMP3, and binding of the antibody PAC-1.

All three chain-lengths of glycopolymers cause platelet aggregation. In **Paper I** the glycopolymer aggregation curves were shown using equal doses (μg/ml). However, if the glycopolymers doses are converted to concentrations (μM), a concentration-chain-length relationship becomes apparent (Figure 3).

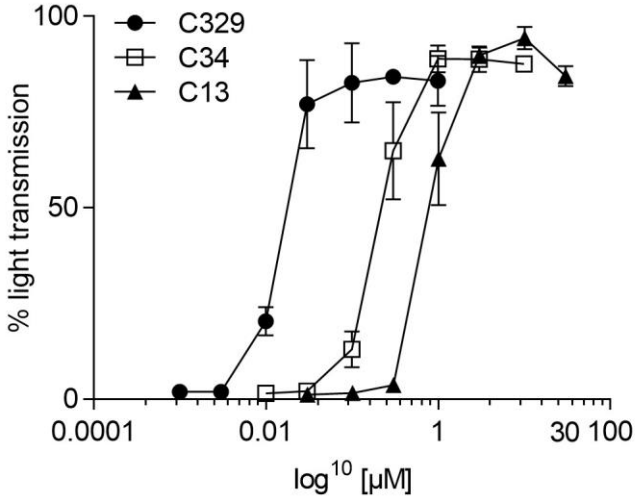

Figure 3 The relationship between concentration and glycopolymer length on platelet aggregation. Isolated human platelets were stimulated with increasing concentrations of C329 (Glycopolymer 3), C34 (Glycopolymer 7), and C13 (Glycopolymer 9). Results are presented as mean increase in light transmission ± SEM (n=3-4).

The concentration/chain-length relationship was published for the first time in **Paper II**, where the longest glycopolymers, C329, and the shortest glycopolymers, C13, were compared. The longer polymer is 20 times more potent at causing platelet aggregation in both human and mouse platelets. However, the mechanism of action of glycopolymers-induced platelet aggregation turned out to be different between the two species.

Sulfated synthetic glycopolymers act via PEAR1/GPIbα/CLEC-2

Paper I revealed that synthetic glycopolymers are platelet activators with fucoidan-mimetic properties. However, the major question remaining unanswered was; which is the molecular mechanism responsible for glycopolymers induced platelet activation?

Due to the multivalent nature of fucoidans, a multitude of molecular inter-actions have been suggested [31]. An interesting study, using *Fucus vesiculosus*, revealed that seaweed polysaccharides may be CLEC-2 agonists [18]. The downstream signalling caused by CLEC-2 is mainly described using the snake venom Rhodocytin [54], and resembles the signalling pathway described for GPVI [55,56]. Hence, in **Paper II,** the downstream signalling target Syk, LAT, and PLCγ2 were investigated and neither were involved in glycopolymer-induced platelet activation. No phosphorylation was detected for any of the downstream phosphorylation targets of CLEC-2 in human platelets. In addition, Syk-inhibition did not result in decreased aggregation to synthetic glyco-polymers. These results suggested that the glycopolymers-induced aggregation was unrelated to CLEC-2 in human platelets.

In the end of 2016, Vandenbriele et al. published the paper titled *"Dextran sulfate triggers platelet aggregation via direct activation of PEAR1"* [29]. Dextran sulfate is another sulfated polysaccharide that mainly consists of glucoside, instead of fucose. Vandenbriele et al. showed that dextran sulfate acts via both CLEC-2 and PEAR1. They detected phosphorylation of PEAR1, and a downstream signalling cascade dependent on PI3K-Akt to cause integrin αIIbβ3 activation [29]. Phosphorylation of PEAR1 is detected at ~ 125 kDa, similar to the rapid and time-dependent phosphorylation we previously observed in **Paper I**. Consequently, further investigation of PEAR1 as a potential target for synthetic glycopolymers and natural fucoidan in human platelets was a natural step. Experiments were performed comparing C329, C13, fucoidan from *Fucus vesiculosus*, and dextran sulfate with rhodocytin, a well-known CLEC-2 agonist [54,56].

In **Paper II** we establish that synthetic glycopolymers and natural polysaccharides cause human platelet aggregation via PEAR1, which becomes phosphorylated within 20 seconds of stimulus. In addition, the aggregation induced via PEAR1 can be inhibited by anti-PEAR1 antibodies. Expression of truncated versions of PEAR1 were used in AVEXIS, where a biotinylated version of the synthetic glycopolymers were tested for interaction with the truncated proteins. EGF-like repeat 1-12, 13, and 12-14 were investigated for binding to synthetic sulfated glycopolymers. The experiments revealed that the EGF-like repeat 13 of PEAR1 is sufficient for binding the synthetic sulfated glycopolymers. The importance of the sulfation and the negative charge was once again established as the non-sulfated glycopolymers did not bind to PEAR1.

Our results show that human platelet aggregation to synthetic glycopolymers and natural fucoidan is PEAR1-dependent. However, the mechanism in mouse turned out to be another. In collaboration with Steve P. Watson's laboratory at the University of Birmingham, we showed that glycopolymers do indeed bind to PEAR1, but in mice the activation takes place via CLEC-2. Synthetic glycopolymers cause phosphorylation of CLEC-2 in wild type mice. In a mouse model lacking CLEC-2, the synthetic glycopolymer-induced aggregation is inhibited, and the shape-change detectable in wild type mouse platelet aggregation is abolished.

In collaboration with Marc F. Hoylaert's laboratory in KU Leuven we confirm that platelet aggregation in mice lacking PEAR1 is inhibited when using low concentrations of the synthetic glycopolymers. However, higher concentrations can overcome the lack of PEAR1, but not the lack of CLEC-2.

Paper II incudes an interesting sub-finding. It is known that fucoidans can interact with thrombin [32], but we wondered if it could interact with GPIbα as well. It has been shown that thrombin induces aggregation via PAR1 and PAR4 with support of GPIbα. Low doses of thrombin can be inhibited using blocking antibodies against GPIbα, which can be bypassed by higher concentrations of thrombin [57]. In **Paper II** the synthetic glycopolymers were tested against two different GPIbα-blocking antibodies; HIP1 and SZ2. Both antibodies reduced but did not abolish the synthetic glycopolymer-induced human platelet aggregation. These findings were later verified in mice lacking the extracellular domain of GPIbα. Platelet aggregation by synthetic glycopolymers is reduced in mice lacking the extracellular domain of GPIbα. However, the prominent CLEC-2-driven shape-change remained.

Sulfated synthetic glycopolymers stimulate a pro-inflammatory response in endothelial cells

In **Paper III** we extend the studies using synthetic glycopolymers and natural fucoidan to cultured human endothelial cells. The aim of **Paper III** was to characterise the effects of synthetic glycopolymers and natural polysaccharides on inflammation mediator synthesis and release from human endothelial cells.

Endothelial cells were exposed to synthetic glycopolymers C329 and C13, fucoidan from *Fucus vesiculosus* or dextran sulfate for 24h or 48h. Cell culture medium and lysates were harvested and analysed by Olink® technology using the Inflammation panel and the Cardiovascular III panel.

Endothelial cells can release a multitude of chemokines and cytokines upon stimulation by substances like lipopolysaccharide (LPS) or TNF-α [58]. The synthetic glycopolymers and natural polysaccharides caused relatively few changes in protein expression and release by HUVECs that did not resemble what can be observed after LPS stimulation [58,59]. After 24h, C-X-C ligand 11 (CXCL11) was the most prominently upregulated protein, and the only statistically significant protein release induced by all four treatments.

After 48h of stimulation the expression of several proteins was affected. In the Cardiovascular III panel, the top upregulation after treatment with synthetic glycopolymers and dextran sulfate was C-C-Motif Chemokine Ligand 2 (CCL2). The protein expression data is described in further detail in **Paper III**.

We decided to perform extended investigations of the two most affected proteins; CXCL11 and CCL2. The upregulation of CXCL11 was confirmed on gene expression level. The increase in RNA was apparent first after 24h, which is similar to what previously has been described for interferon-induction of CXCL11 in endothelial cells [60].

In addition to the Olink® analysis, CXCL11 and CCL2 protein levels were analysed using conventional ELISA techniques. A dose-response series was analysed for both proteins. The CCL2 ELISA revealed a similar expression pattern of the synthetic glycopolymers compared to the Olink® analysis on cell lysates. Dextran sulfate induced a 2.4-fold increase in CCL2 compared to control, as measured by Olink®. However, dextran sulfate-induced CCL2 release was not detectable using the ELISA. In the CXCL11 ELISA the results showed a reverse protein-dose relationship. Fucoidan have previously been shown to bind to various cytokines [61]. Hence, we investigated if synthetic

glycopolymers may interfere with the ELISA. CXCL11-standard was preincubated with increasing doses of C329 and there is a dose-dependent interference with the CXCL11-antibody binding of the ELISA.

Glycosaminoglycans (GAGs) are known to interact with chemokines and cytokines [61,62]. Famous examples are heparin, and heparan sulfates [62,63]. Sulfated GAGs are modifiers of chemokine function, they can increase the chemokine dimerisation or modify chemokine-receptor interactions. Endothelial cell surface GAGs bind chemokines promoting their presentation to immune cells [62,64]. The interaction between GAGs, chemokines and cytokines is charge-dependent. The sulfation of the GAGs and their negative charge is necessary to interact with clusters of positive amino acids on chemokines and cytokines. There are three amino acids with positive charge: arginine, lysine, and histidine. Chemokines generally contain large numbers of positively charged amino acids [62–64]. The chemokines CXCL11 and CCL2 contain 16 of 73 and 14 of 74 positively charged amino acids respectively [63,65]. Sequences like BBXB and BBXXB (where B is a positive amino acid) have been proposed as sites of GAG-interactions [62,63]. Interestingly, PEAR1 EGF-like repeat 13 contains a BXXBB sequence [66], in similar to CXCL11, which contains both a BBXB and a BBXXBB sequence [63]. Both proteins seem to interact with synthetic glycopolymers. Dextran sulfate on the other hand seems to interfere with CCL2-antibody binding in commercial ELISAs. The chemokine CCL2 does not contain either one of the consensus binding sequences [65], and neither does it seem to interfere with any protein-analysis in the presence of α-L-fucoside pendant glycopolymers. A study by Severin et al. shows that the BXBB or BXXBB sequences of CXCL11 are not vital for heparin binding. Instead a BXBXXB sequence seems essential for the heparin binding [63]. **Paper II** concludes that GPIbα is of importance for platelet aggregation by synthetic glycopolymers. There are studies performing sequence alignment of GPIbα [67], a BXBXXB sequence can be found in the macroglycopeptide region. The two antibodies that were used for evaluating the participation of GPIbα in human platelet aggregation bind to other regions of the receptor. The antibody clones SZ2 and HIP1 bind to the anionic region, and the leucine-rich repeat region respectively [68]. Both antibodies reduced but did not abolish aggregation to synthetic glycopolymers.

It is important to mention that there is more to glycoprotein- and polysaccharide-protein-interactions than merely amino acid sequence. For example, Salanga et al. suggest that multiple GAG binding sites on the protein can increase the affinity together [65]. Protein tertiary structure and the 3D positioning of positively charged clusters most likely plays a critical in binding and interaction.

A novel class of purine analogues inhibit Rho-associated kinases

Paper IV has similarities in the scientific approach compared to **Paper I** and **Paper II**. We utilised a unique synthetic construct with a biological effect of unknown mechanism. However, the construct used **in Paper IV** is of a completely different chemical class. **Paper IV** aimed to investigate the effect of a piperazine purine with a nitrate ester motif, named MK128, on platelet function and to elucidate the mechanism of action. The compound has previously been shown to be anti-inflammatory and cardioprotective [43].

The effect of MK128 was evaluated on PAR1-, GPVI-, and TPα-mediated aggregation. MK128 reversed aggregation to SFLLRN (single amino acid code), and reduced secretion. Collagen-induced GPVI-mediated platelet aggregation and secretion was strongly reduced by MK128 while aggregation and secretion induced by the stable thromboxane A2 analogue U46619 was nearly abolished.

Since the effect of MK128 was observed on platelet functions initiated via several signalling pathways, we hypothesised that MK128 may interfere with the secondary feedback loops of platelet activation.

MK128 is a purine analogue and may therefore interact with purine receptors or purine binding domains of other proteins. Measurement of cytosolic calcium elevation using P2Y12, P2Y1, and P2X1 receptor agonists in the presence or absence of MK128 did not point to purinergic receptor antagonism. Thromboxane A2-mediated cytosolic calcium elevation was affected, but the magnitude of inhibition was small. Collectively, this shows that calcium signalling is not the main target of inhibition by MK128.

MK128 contains a nitrate ester motif and has previously been proposed as a potential nitric oxide donor [43]. Platelets are highly sensitive to nitric oxide-induced cGMP-elevation. For example, nitric oxide donors like SNAP completely abolishes platelet aggregation by many activators. A soluble guanylyl cyclase inhibitor, NS2028, completely reversed SNAP-induced platelet inhibition. However, NS2028 did not reverse MK128-induced platelet inhibition, indicating for a mechanism unrelated to nitric oxide. This was further supported by the findings that cGMP or cAMP-dependent phosphorylation sites on VASP were unaffected by MK128.

The effect of MK128 was further evaluated on signalling-related kinases using FRET-based kinase screening assay. The kinase analysis revealed that MK128 concentration-dependently inhibits Rho-associated Protein-Kinase (ROCK), a kinase downstream of TPα. In addition, MK128 reduces U46619-induced ROCK-dependent phosphorylation site of the myosin phosphatase subunit (MYPT). The results indicate that the platelet inhibiting effect of MK128 is mainly due to ROCK-inhibition. However, it must be mentioned that the concentration required of MK128 to produce significant inhibition is relatively high, being most efficient at 100μM. Due to the high dose required, we cannot exclude other unspecific interactions by MK128.

Summary of papers

Paper I

Synthetic sulfated α-L-fucoside pendant glycopolymers have fucoidan-mimetic properties. Both long and short synthetic glycopolymers cause human platelet aggregation, intracellular phosphorylation, and surface protein changes in similarity to natural fucoidan from *Fucus vesiculosus.*

Paper II

Synthetic sulfated α-L-fucoside pendant glycopolymers bind directly to, and cause human platelet aggregation via, PEAR1. EGF-like repeat 13 is sufficient to bind synthetic sulfated glycopolymers. Mouse platelet aggregation to synthetic glycopolymers is induced mainly through CLEC-2, whereas PEAR1 may play a minor role. The aggregation is supported by GPIbα in both species.

Paper III

Synthetic sulfated α-L-fucoside pendant glycopolymers and natural polysaccharides affect protein levels in both cell lysates and cell culture medium of human vascular endothelial cells. Data analysis reveal that the protein release after 24h is pro-inflammatory. The top protein upregulation detected by all treatments was CXCL11.

Paper IV

Synthetic piperazine-purine with nitrate ester motif blocks platelet aggregation and secretion mainly via dose-dependent inhibition of ROCK, downstream of TPα. The purine analogue does not influence on NO/cGMP-signalling and does not interfere with purinergic platelet receptors.

Conclusion

The findings presented in this thesis show that synthetic constructs can be valuable tools for studying biological functions. This study uses two types of synthetic constructs, sulfated glycopolymers and a purine analogue with a nitrate ester motif. Both constructs are biologically active, with two completely different mechanisms of action.

This thesis concludes that:

- Synthetic glycopolymers cause length- and sulfation-dependent human and mouse platelet aggregation.

- Synthetic sulfated glycopolymers cause human platelet activation via PEAR1, mouse platelet activation via CLEC-2, and in both species the activation is supported by GPIbα (Figure 4).

- Synthetic sulfated glycopolymers cause a pro-inflammatory profile in cultured human endothelial cells and are potentially being able to interact with chemokines.

- A nitrate ester motif bearing purine analogue is a ROCK inhibitor that reduces platelet aggregation and secretion.

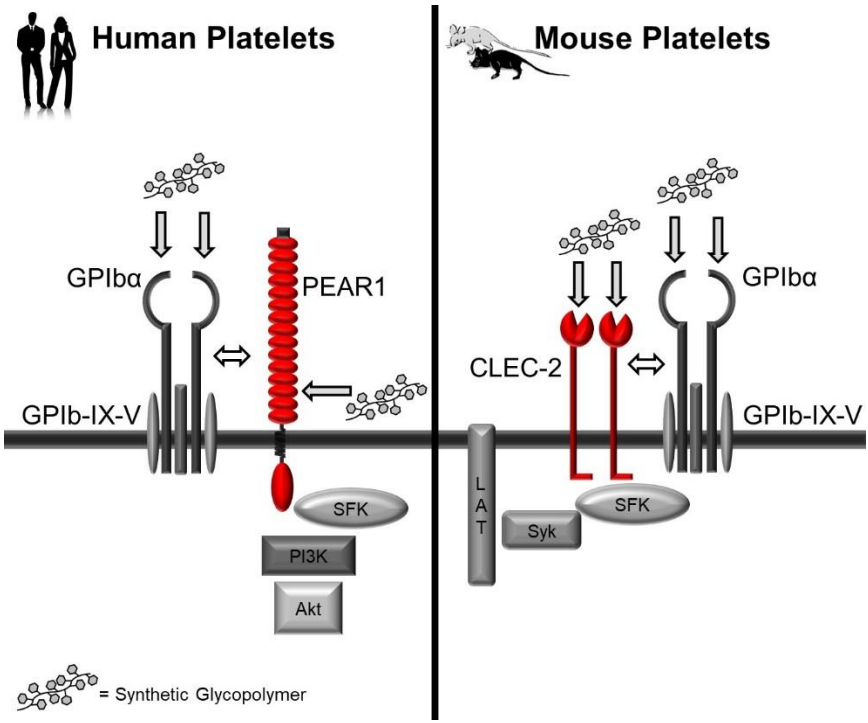

Figure 4 Visualisation of differences between human and mice in terms of synthetic glycopolymer- and natural polysaccharide-induced platelet aggregation as shown in ***Paper II*** *of the thesis. Used with permission from Blood Advances and the Sheridan Publishing Group.*

Future perspectives

This thesis has shown that synthetic sulfated α-L-fucoside pendant glycopolymers are biologically active and cause activation in human platelets via PEAR1. In **Paper II** we speculate that the PEAR1 activation mechanism takes place via clustering. Potential clustering mechanisms of PEAR1 should be further explored by using high technological imaging techniques.

Previous studies have shown that the high-affinity immunoglobulin E receptor is a potential native ligand for PEAR1 [69]. The high-affinity immunoglobulin E receptor is a highly glycosylated protein [70] which binds to the same region of PEAR1 as synthetic glycopolymers do. This indicates that the synthetic glycopolymers can be used as tools for studying the physiological role of PEAR1, which remains to be further explored.

There are studies indicating for altered platelet responses to various agonists in individuals with specific PEAR1 mutations [71–74]. However, these studies evaluated stimuli via other receptors, such as GPVI or P2Y12, rather than PEAR1 itself. In this case, the synthetic glycopolymers may be used for investigating the PEAR1 functionality in individuals with various PEAR1 mutations.

PEAR1 is expressed on both platelets and endothelial cells [66]. Previous studies performing knock-down of PEAR1 in cultured endothelial cells have shown alterations in angiogenesis [75] and mitochondrial function [76]. In **Paper III** we show that synthetic glycopolymers and natural polysaccharides induce expression of the interferon-related chemokine CXCL11. However, we did not explore the role of PEAR1 in the observed protein release. Endothelial cell knock-down of PEAR1 in combination with stimulation using the PEAR1-binding constructs may provide further knowledge on the physiological role of PEAR1. Further explorations of a potential role of PEAR1 in innate immunity may be warranted.

The thesis also concluded that the synthetic purine analogue bearing a nitrate ester motif supress platelet functions via ROCK-inhibition. In a larger context, MK128 is merely the first generation of these constructs. A second generation of these purine analogues have been designed and are currently being evaluated by us and our colleagues. The study of MK128 shed valuable light on which readouts that should be measured when evaluating the efficacy of the next generation of these drugs. Hopefully, the next generation of nitrate ester bearing purine analogues can reach a higher selectivity, and specificity than its predecessor.

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