THE VASCULAR EFFECTS OF CLOPIDOGREL

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Pharmacology and Toxicology- Environmental Toxicology – Doctor of Philosophy

2022

ABSTRACT

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Dual antiplatelet therapy using aspirin with a purinergic receptor $2Y_{12}$ ($P2Y_{12}$) antagonist is the most common preventative method for arterial thrombosis. The favored $P2Y_{12}$ antagonist clopidogrel is a prodrug that is absorbed in the intestine and then converted into the active metabolite by cytochrome P450 enzymes (CYP450s). However, only about 5% of clopidogrel is converted into this active metabolite. The remainder is converted into more than 15 additional metabolites to which the effects have been previously thought inactive. Antiplatelet agents, such as clopidogrel, cause an increased risk of developing cerebral microbleeds and intracerebral hemorrhage. Cerebral blood flow regulation is essential in maintaining blood-brain barrier homeostasis. Larger arteries, like the middle cerebral artery and posterior cerebral artery, generate myogenic tone to regulate cerebral blood flow. Myogenic tone is the contraction of vascular smooth muscle cells in response to increasing pressure. Tone generation protects the capillary bed from changes in pressure that could lead to cerebral microbleeds and intracerebral hemorrhage. When pressure increases in cerebral arteries, these vessels constrict to decrease blood flow. Arteries also undergo active contraction when damaged to reduce blood flow. Both acute vasoconstriction (myogenic tone) and chronic vasoconstriction (in response to injury) are crucial in the prevention and protection of arteries from major bleeds. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ are expressed in the endothelium of cerebral arteries, while $P2Y_2$, $P2Y_4$, and $P2Y_6$ are expressed in the smooth muscle. $P2Y_4$ and $P2Y_6$ in the smooth muscle are key regulators of myogenic tone. Due to the structural similarity between purinergic receptors, additional clopidogrel metabolites might modulate $P2Y_1$, $P2Y_2$, $P2Y_4$, or $P2Y_6$ on the vasculature.

A major focus of the work described in this dissertation was to identify which receptor(s) clopidogrel or its metabolites inhibit. My studies tested the hypothesis that clopidogrel

metabolites modulate $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and/or $P2Y_{14}$ signaling in the vasculature which results in increased bleeding side effects. I addressed this hypothesis using pharmacological and genetic approaches. My studies show that clopidogrel inhibits ADP-induced platelet aggregation, but this effect does not correlate with the increase in adverse bleeding. Additionally, my studies demonstrate endothelial $P2Y_2$-mediated vasoconstriction is inhibited in clopidogrel-treated rabbits. Furthermore, the prodrug clopidogrel, the M1 metabolite, and the M2 metabolite are not responsible for inhibiting $P2Y_2$ signaling. Lastly, I discovered both $P2Y_{12}^{-/-}$ mice and clopidogrel-treated mice have decreased distensibility and increased arterial stiffness. My studies could have wide implications for the development of new antiplatelet agents. The finding of $P2Y_2$ inhibition by clopidogrel allowed us to better define the mechanism to which clopidogrel causes adverse bleeding. This provides a basis for what to avoid in the development of improved antiplatelet therapies.

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To My Family

ACKNOWLEDGMENTS

My dissertation work would not have been possible without the guidance and support of many people. The most instrumental person in this experience has been my Ph.D. mentor, Dr. Adam Lauver. Dr. Lauver is driven to produce scientists who will make an impact on the world. He taught me the basics of platelet research and provided an environment in which I could grow into the scientist I am today. His passion for science and mentorship paved the path for me to become a well-versed scientist. Thank you for inspiring me to do more than I thought capable.

I would also like to emphasize the gratitude I have towards my co-mentor, Dr. Anne Dorrance. She was the first to introduce me to cerebral vessel research which turned out to be the bulk of my dissertation work. Although in the beginning, I had little desire to study cerebral vessels, she made the research exciting and continually reminded me of the impact this work would provide. Thank you for your guidance and support.

Thank you to my committee members, Dr. James Luyendyk and Dr. Robert Roth. You both asked the tough questions that stimulated me to dig deeper. Your guidance was crucial in my ability to communicate science effectively.

To my lab members, Barbara Christian and Nick Ether, thank you for helping me in the day-to-day. Barbara was more than a lab member; she will be a lifelong friend. She was in it for every moment of my Ph.D. She assisted me in my experiments, read over my papers, helped me practice my presentations, and listened when I needed someone to vent to. I will forever cherish our 3:45 pm dance parties. In the toughest of times, that is what lifted my spirits. Nick and I started our Ph.D.'s the same year and have gone through every step together: our first talks, our proposal defenses, the struggle of the COVID pandemic in the middle of our Ph.D., and now the completion of our dissertation work. While our studies are distinct, having someone by your side for the journey is comforting. I also want to thank the 14 students (DVM, undergraduate, graduate) who I have mentored throughout my Ph.D. The enthusiasm you all

had for learning and the brightness in your eyes reminded me of the joy science brings me. I have become a much better mentor because of all of you. I hope I passed on my love for science and truly believe each one of you will go on to do amazing things.

The Department of Pharmacology and Toxicology is filled with positive, insightful members. The ability to collaborate with any lab allowed for my growth as a scientist. I am appreciative of everyone in this department as they all showed their desire for me to succeed. The office and support staff were essential to this research through their contribution of grant applications, academic support, and desire to make work a fun environment. The Institute of Integrative Toxicology expanded my knowledge base and allowed for an outside perspective of my work. Thank you for your guidance throughout my graduate career.

This work would not have been possible without the F31 Fellowship funding I received through the National Heart, Lung, and Blood Institute at the National Institutes of Health. Funding through the Department of Pharmacology and Toxicology as well as the Institute of Integrative Toxicology have also been important financial contributors to the work of this thesis. Thank you to all my funding sources.

I could not have achieved this without the support I received from my family. Thank you to my parents, Pat and Connie Henderlong, for providing me the foundation to achieve anything I put my mind to. To my siblings, Dan, Diana, and David, for pushing me to do more and always reminding me of who I am. To my in-laws, Mike, Lorie, and Kate Kuszynski, for making me feel important to this world. To my dogs for being the emotional support I needed. Finally, to my beloved husband, Matt Kuszynski, thank you for your unending, unconditional support. I truly could not have done this without you by my side.

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KEY TO ABBREVIATIONS

2MeSADP	2-(methylthio)adenosine 5'-diphosphate
AA	arachidonic acid
ADP	adenosine diphosphate
Ang II	angiotensin II
ANOVA	analysis of variance
CES1	carboxylic esterase 1
CYP450s	cytochrome P450s
DAPT	dual antiplatelet therapy
DIO	diet-induced obese
DMA	N, N-dimethylacetamide
EDCF	endothelium-derived contracting factors
EGTA	ethylene glycol tetra acetic acid
ERK	extracellular-signal-regulated kinase
FITC	fluorescein isothiocyanate
GPCR	G-protein coupled receptor
GSH	glutathione
H_2S	hydrogen sulfide
H4	active metabolite of clopidogrel
IL-1R$^{-/-}$	IL-1 receptor knockout
IM	intramuscular
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MCA	middle cerebral artery
MCP-1	monocyte chemotactic protein-1
MEK	mitogen-activated protein kinase
MyD88	myeloid differentiation primary response protein 88
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOX	NADPH oxidase
$P2Y_{12}$	purinergic receptor $2Y_{12}$
$P2Y_{12}^{-/-}$	$P2Y_{12}$ knockout

PCA	posterior cerebral artery
PEG400	polyethyleneglycol-400
PLATO	Platelet Inhibition and Patient Outcomes
PON1	paraoxonase 1
PPP	platelet-poor plasma
PRP	platelet-rich plasma
PSS	physiological salt solution
ROS	reactive oxygen species
SNP	sodium nitroprusside
SR26334	carboxylic acid metabolite of clopidogrel
Up_4A	uridine adenosine tetraphosphate
UTP	uridine triphosphate
VCAM-1	vascular cell adhesion molecule-1
VSMC	vascular smooth muscle cells
WBC	white blood cell

CHAPTER 1

Introduction

Part 1

Pleiotropic Effects of Clopidogrel

Modified from: Kuszynski DS, Lauver DA. Pleiotropic effects of clopidogrel. Purinergic Signal. 2022 Jun 9. doi: 10.1007/s11302-022-09876-0. Epub ahead of print. PMID: 35678974.

Abstract

Clopidogrel is a widely prescribed prodrug with antithrombotic activity through irreversible inhibition of the $P2Y_{12}$ receptor on platelets. It is FDA approved for the clinical management of thrombotic diseases like unstable angina, myocardial infarction, stroke, and during percutaneous coronary interventions. Hepatic clopidogrel metabolism generates several distinct metabolites. Only one of these metabolites is responsible for inhibiting the platelet $P2Y_{12}$ receptor. Importantly, various non-hemostatic effects of clopidogrel therapy have been described. These non-hemostatic effects are perhaps unsurprising as $P2Y_{12}$ expression has been reported in multiple tissues, including osteoblasts, leukocytes, as well as vascular endothelium, and vascular smooth muscle. While the “inactive” metabolites have been commonly thought to be biologically inert, recent findings have uncovered $P2Y_{12}$-independent effects of clopidogrel treatment that may be mediated by understudied metabolites. In this chapter, I summarize both the $P2Y_{12}$-mediated and non-$P2Y_{12}$-mediated effects of clopidogrel and its metabolites in various tissues.

1. Introduction

Platelets are activated by several endogenous chemical mediators, including adenosine diphosphate (ADP), thrombin, and thromboxane. One therapeutic strategy to inhibit the pathological action of platelets in thrombotic diseases is selective inhibition of one specific receptor pathway, such as ADP-induced activation of the purinergic receptor $2Y_{12}$ ($P2Y_{12}$). $P2Y_{12}$ is a G-protein coupled receptor (GPCR) that drives platelet activation. Activation of $P2Y_{12}$ indirectly increases cytoplasmic calcium to cause platelet shape change and aggregation. Additionally, activation of platelets by agonists such as collagen and arachidonic acid causes secretion of dense granules that result in subsequent activation of $P2Y_{12}$, thereby potentiating aggregation. Therefore, it is crucial to inhibit this receptor. However, it is common to combine therapeutic agents to inhibit multiple platelet activation pathways. Dual antiplatelet therapy (DAPT) is commonly used for the treatment of thrombotic diseases and involves the combined administration of low-dose aspirin, which inhibits the cyclooxygenase-dependent activation pathway, with a $P2Y_{12}$ antagonist. Clopidogrel, a thienopyridine antiplatelet agent, is the favored $P2Y_{12}$ antagonist for long-term DAPT. Indications for its use include percutaneous coronary intervention (balloon angioplasty and stent implantation), acute coronary syndrome (ACS), and secondary prevention post-coronary artery bypass graft.

An earlier thienopyridine antiplatelet agent, ticlopidine, was associated with severe hematological side effects including leucopenia and thrombocytopenia. To address these adverse effects, thousands of analogs of ticlopidine were generated in the hope of identifying novel compounds with improved risk/benefit profiles. One such analog, clopidogrel, began preclinical evaluation in 1987 with ultimate approval for use in the United States coming in 1997 and a worldwide launch in 1998 [1]. Interestingly, at the time of its approval, the molecular target of clopidogrel was unknown, although its action was known to be unique from aspirin, sulfinpyrazone, and dipyridamole. Subsequently, it was recognized that the compound was a potent inhibitor of ADP-mediated platelet aggregation [2]. The discovery of the $P2Y_{12}$ receptor

in 2000 [1,3,4] paved the way for its identification as the primary pharmacologic target of clopidogrel in 2001 [5].

Clopidogrel is a prodrug that requires hepatic bioactivation to generate the active metabolite responsible for inhibiting platelets [6]. The active metabolite of clopidogrel (M13) acts by covalently modifying cysteine residues within the ligand-binding domain (Cys17 and Cys270) of $P2Y_{12}$ [7]. Interestingly, the identity of M13 and the enzymes (CYP450s) responsible for its formation were a mystery at the time of clopidogrel's approval. The clinical pharmacokinetics of clopidogrel were determined using the plasma concentration of the primary circulating metabolite (SR26334) as a surrogate [8]. This carboxylic acid derivative is a product of esterase-dependent metabolism and is not responsible for inhibiting platelet aggregation. The chemical structure of the active metabolite was characterized in 2000 and the important enzymes involved in its formation were systematically identified with the last one proposed in 2011 [1,9,10]. The pharmacology of SR26334 and the other clopidogrel metabolites has not been extensively investigated, although ample evidence suggests meaningful biological effects of these abundant metabolites.

2. Clopidogrel Metabolism

To accelerate the antiplatelet effects, clopidogrel is prescribed at an initial loading dose of 300 mg followed by a maintenance dose of 75 mg/day. Clopidogrel is readily absorbed in the intestine and then converted into several distinct metabolites by a variety of metabolic enzymes, including carboxylic esterase 1 (CES1), members of the CYP450 family, and paraoxonase 1 (PON1). Approximately 85% of the prodrug is converted into SR26334 by CES1, while only about 5% of the prodrug is ultimately converted into M13 by a CYP450-mediated process (Figure 1). The enzymes CYP2C19 and CYP3A4 are critically important for the catalysis of these reactions [15–19]. While it is clear that M13 is responsible for inhibiting

the $P2Y_{12}$ receptor on platelets, the biological effect(s) of the other metabolic products remain unknown.

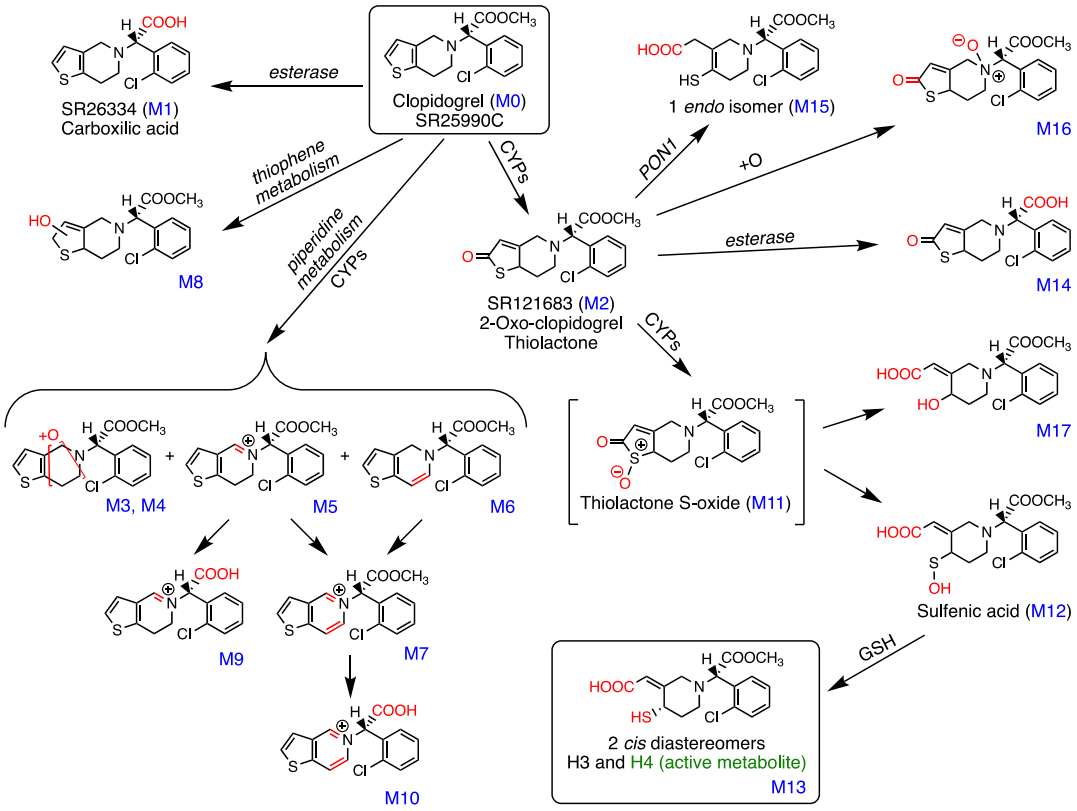

Figure 1: Proposed bioactivation pathway of clopidogrel. The proposed bioactivation pathway of the clopidogrel prodrug is dependent upon a complicated, multistep, enzyme-dependent metabolic process. The majority of the ingested prodrug is hydrolyzed by esterases (including CES1) to form the carboxylic acid metabolite (SR26334). The remaining clopidogrel is metabolized by CYP450 enzymes (primarily CYP2C19 and CYP3A4), producing various chemical products including M13, the metabolite responsible for inhibiting $P2Y_{12}$. Metabolism information is summarized from previous reports [1,11–14].

The complex nature of clopidogrel metabolism has significant potential to induce variation in patient responses. For example, a decrease in the antiplatelet activity is observed in patients with loss of function polymorphisms in CYP2C19 (*2 and *3 alleles) [20–22]. Patients with CYP2C19 polymorphisms have decreased M13 concentrations and increased SR26334 concentrations [21]. Insufficient clopidogrel conversion to M13 results in poor clinical response, and therefore CYP2C19 loss of function carriers have limited protection from

thrombotic events when treated with clopidogrel compared to individuals with normal enzyme function [21]. Due to interpatient variability and the potential for lack of clinical response, the United States Food and Drug Administration added a *Black Box Warning* to (1) warn patients about the reduced effectiveness for those who do not effectively metabolize the prodrug, (2) inform clinicians to evaluate patients for CYP2C19 activity, and (3) instruct clinicians to select other antiplatelet therapeutics for those who do not effectively metabolize clopidogrel [23].

The response of clopidogrel is further complicated by interactions with other drugs. CES1, CYP2C19, and CYP3A4 are commonly involved with the metabolism of other pharmaceuticals. Therefore, induction or inhibition of these enzymes by other pharmaceutical agents could affect the bioactivation and clinical response of clopidogrel. For instance, omeprazole, a commonly prescribed proton pump inhibitor used in the treatment of gastroesophageal reflux disease, competitively inhibits CYP2C19 and, in doing so, decreases the bioactivation of clopidogrel [20,24]. Ethanol consumption also impacts clopidogrel metabolism by increasing the formation of the M13 metabolite thereby increasing platelet inhibition [25]. In turn, there is decreased SR26334 metabolite concentration with ethanol (3 g/kg) consumption which appears to result from a shift from the CES1 metabolic pathway toward CYP450-mediated metabolism [26].

Type II diabetes and insulin resistance have been identified as risk factors for diminished clopidogrel response. Generation of M13 is decreased by 40% in diabetic patients compared to non-diabetic patients [27–29]. Diet-induced obese (DIO) mice, which are a useful model of human Type II diabetes, have a diminished response to the clopidogrel prodrug yet respond normally to a conjugate of the active metabolite that does not require enzymatic activation [30]. Interestingly, DIO IL-1 receptor knockout ($IL\text{-}1R^{-/-}$) mice were able to overcome the clopidogrel resistance as a result of increased CYP450 expression and therefore increased M13 generation [30]. Ultimately, it appears diabetes downregulates CYP2C19, resulting in reduced formation of M13 and diminished antiplatelet effects.

Most of the metabolites of clopidogrel are electrophilic species that may unselectively bind to cellular and circulating macromolecules. While not all binding events lead to damaging biological effects, previous reports indicate these reactive metabolites can cause various effects. The primary objective of this chapter is to present a comprehensive list of all the reported off-target effects of clopidogrel.

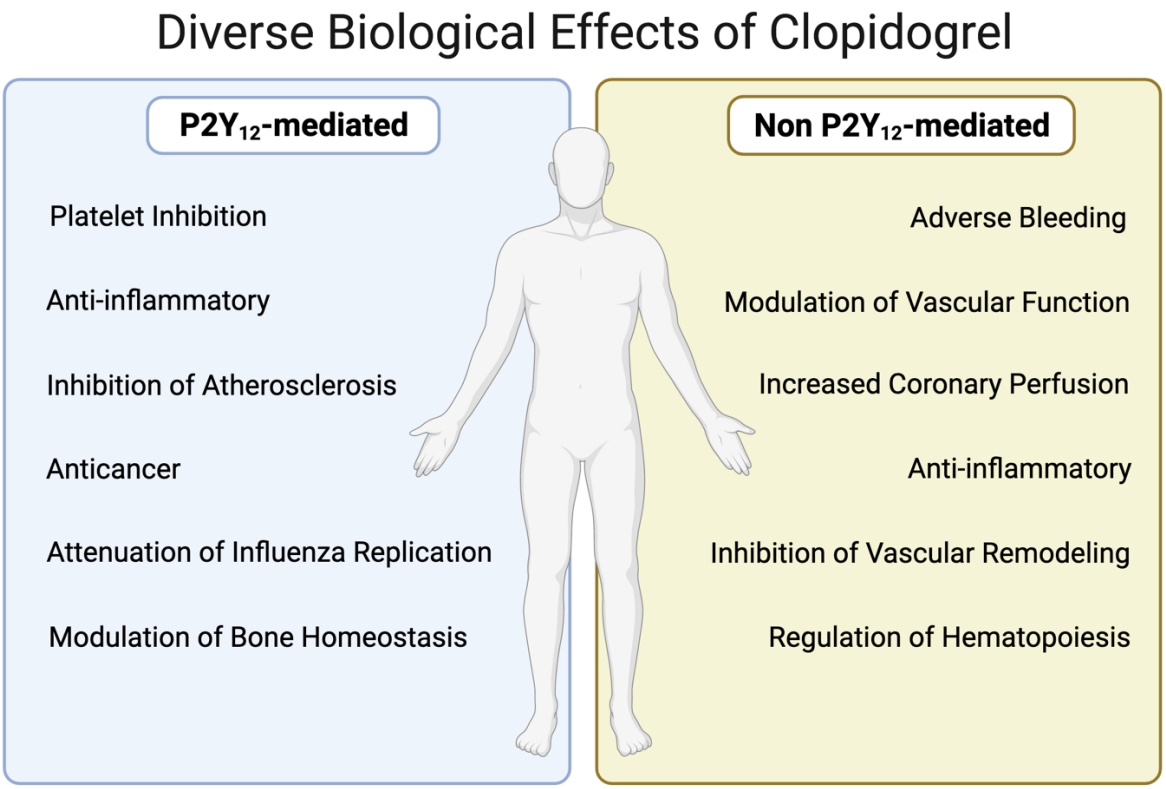

Figure 2: Diverse biological effects of clopidogrel and its metabolites. Clopidogrel effects have been described in various tissues in both $P2Y_{12}$-dependent and -independent manners.

3. Off-target Clopidogrel Effects

3.1 Non-Hemostatic $P2Y_{12}$-Mediated Effects of Clopidogrel

Clopidogrel treatment is prescribed for the inhibition of the $P2Y_{12}$ receptor on platelets to prevent activation and aggregation. While efficacious in that mechanism, several other non-hemostatic $P2Y_{12}$ effects have been described. Platelets interact with leukocytes and

endothelial cells during stress and inflammation [31]. Additionally, platelets express Toll-like receptors, allowing for interactions with neutrophils and monocytes to initiate immune responses [31]. $P2Y_{12}$ is also suggested to be expressed in microglia [32–34], smooth muscle, and endothelial cells [35–38]. Furthermore, $P2Y_{12}$ is putatively expressed in several other tissues including the brain, reproductive organs, thyroid, lung, adrenal gland, tongue, esophagus, kidney, liver, colon, bladder, heart, skin, spleen, lymph node, pituitary gland, retina, salivary gland, stomach, gull bladder, adipose tissue, tonsil, appendix, and bone marrow [39]. However, it is important to note that tissue-specific expression is difficult to rigorously demonstrate as samples are often contaminated with platelets. Adequate antibodies for $P2Y_{12}$ do not exist, therefore only RT-PCR is useful for investigating receptor expression. While the focus of clopidogrel actions has been on platelets, the proposed broad tissue-specific expression of $P2Y_{12}$ likely enables multiple mechanisms whereby clopidogrel treatment mediates non-hemostatic $P2Y_{12}$ effects. Due to the complex interactions platelets have with other circulating cells and the vessel wall, as well as the reputed broad expression of $P2Y_{12}$ within the body, it is difficult to distinguish which clopidogrel effects are mediated by local $P2Y_{12}$ inhibition versus indirect effects of platelet inhibition.

3.1.1 Modulation of atherogenesis and the progression of atherosclerosis

Atherosclerosis is an immunoinflammatory disease of medium to large arteries that results in the deposition of fatty plaques on the artery wall. Atherosclerosis is not life-threatening itself, but occlusive thrombus formation due to plaque rupture can induce downstream ischemia resulting in unstable angina, stroke, or myocardial infarction. Endothelial cells, leukocytes, and smooth muscle cells are all cellular mediators of atherosclerosis. Lipoprotein particles penetrate the endothelial layer into the subendothelial space, where they become pro-inflammatory. The endothelium is then activated by inflammatory cytokines to express adhesion molecules that recruit blood-borne cells to the atherosclerotic lesion. This inflammation leads to the recruitment of monocytes and T-lymphocytes [40]. As the disease

progresses, intimal smooth muscle cells heal and repair the arterial injury. Smooth muscle cells stabilize the plaque, decreasing the chance of rupture but also narrowing the vascular lumen, thereby reducing blood flow [40,41].

Several studies have demonstrated clopidogrel's ability to reduce atherosclerosis [42–46]. Activated platelets release platelet-derived growth factor, which causes the secretion of matrix metalloproteinase-2. Matrix metalloproteinases degrade several extracellular matrix proteins to promote inflammation [47]. Platelets also induce monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1) expression in endothelial cells, which initiates monocyte recruitment and plays a role in atherosclerotic lesion formation [48,49]. Indeed, patients receiving clopidogrel have a significant reduction in atherosclerotic plaque inflammation [50]. Clopidogrel has also been reported to significantly reduce atherosclerotic lesion formation and reduce the inflammatory response in ApoE-deficient mice, a useful animal model for understanding the pathophysiology of atherosclerosis [42,51]. Bone marrow transplants were performed in $P2Y_{12}$ knockout ($P2Y_{12}^{-/-}$) mice to differentiate between vessel wall or platelet-derived effects. Atherosclerotic lesions were reduced in vessel wall depleted $P2Y_{12}$, indicating vessel wall $P2Y_{12}$ is involved in the development of atherosclerosis [52]. In a rabbit model of atherosclerosis, animals were fed a high cholesterol diet followed by balloon injury to the iliac artery. The rabbits were treated with clopidogrel throughout the study and monitored for the development of atherosclerosis. Treatment with clopidogrel significantly reduced vascular inflammation and atherosclerotic lesion formation while decreasing the expression of P-selectin, intracellular adhesion molecule-1, VCAM-1, and MCP-1 [43].

3.1.2 Inhibition of hypertension-associated inflammation

Angiotensin II (Ang II) activates the angiotensin type 1 receptor and upregulates Toll-like receptor 4. This activates the myeloid differentiation primary response protein 88 (MyD88) and mitogen-activated protein kinase (MAPK). MAPK activates the nuclear factor kappa-light-

chain-enhancer of activated B cells (NF-kB), resulting in a release of pro-inflammatory mediators. Ang II also causes the oxidation of NADPH to generate reactive oxygen species (ROS) [53]. Moreover, Ang II-mediated inflammation induces platelet activation and subsequent platelet-monocyte binding leading to monocyte activation. Platelet-monocyte binding enhances vascular inflammatory responses [54]. Both an increase in pro-inflammatory mediators and the generation of ROS lead to hypertension. Therefore, chronic Ang II administration is commonly used as a model of hypertension in rats and mice. Since platelets are involved in Ang II-associated hypertension, the inhibition of platelets was assessed with clopidogrel treatment. Phenylephrine contraction and acetylcholine relaxation were impaired in mesenteric arteries from Ang II mice and rats. Interestingly, clopidogrel treatment prevented these effects in an endothelium-dependent manner [54–56]. Additionally, clopidogrel treatment improved the structure of hypertensive arteries. Chronic Ang II-treated mice exhibit vascular remodeling and increased artery stiffness due to elevated arterial pressure. Aortas collected from these animals have increased wall thickness, increased wall-to-lumen ratio, and exhibit impaired vasodilation. Clopidogrel treatment decreased the hypertension-associated changes in aortic structure [54,56]. Ang II-treated mice also develop increased vascular oxidative stress, an effect that is completely abolished by clopidogrel treatment. Vascular NADPH oxidase (NOX)1, NOX2, and NOX4 mRNA and protein levels are increased with Ang II treatment, and concomitant treatment with clopidogrel decreases these levels [54,56]. The beneficial effects of clopidogrel in hypertension are likely due to reduced infiltration of macrophages in the aorta since macrophages are the main source of ROS in vessels, as well as the decreased platelet-monocyte binding [54,57].

3.1.3 Inhibition of angiogenesis

The healing of gastric ulcers requires cell proliferation and angiogenesis. Ischemic tissues release leukotriene B to attract leukocytes and macrophages. These cells phagocytize the necrotic tissue and release pro-inflammatory cytokines to activate fibroblasts, endothelial

cells, and epithelial cells [58]. As a result, endothelial cells migrate, proliferate, and re-establish the microvascular network [59]. In an experimental model, rats were subjected to luminal application of acetic acid to induce gastric ulcers. Daily clopidogrel therapy was initiated in these animals, and ulcer healing was observed. Clopidogrel treatment increased ulcer size, therefore delaying gastric ulcer healing. Moreover, clopidogrel decreased the number of microvessels at the ulcer base. Furthermore, protein and mRNA expression of several angiogenic growth factors (vWF, FGFR2, VEGF, VEGFR2, PDGFRA, and pERK) were significantly decreased with clopidogrel treatment, and angiogenesis was reduced. These findings suggest clopidogrel inhibits angiogenesis by inhibiting the VEGF-VEGFR2-ERK signaling transduction pathway [60]. This effect of clopidogrel could be partially explained by the inhibition of platelet activation thereby leading to a reduction in the release of platelet-derived growth factors, however, further studies are required to determine the exact mechanism of action.

3.1.4 Inhibition of Ras/Raf/MEK/ERK signaling pathway

Activation of the $P2Y_{12}$ receptor in human lung epithelial (A549) cells leads to Ras/Raf/Mitogen-activated protein kinase (MEK)/extracellular-signal-regulated kinase (ERK) signaling, and inhibition of that pathway has been linked to various biological effects [61–63]. The Ras/Raf/MEK/ERK signaling pathway allows for communication between cell surface receptors and downstream transcription factors, which induce cellular proliferation, differentiation, and survival. Several RNA viruses induce the Ras/Raf/MEK/ERK signaling to potentiate their replication [64]. Clopidogrel was examined as a novel treatment for influenza since it targets the $P2Y_{12}$ receptor. Calu-3 human bronchial epithelium cells, a common cell line used to study influenza infection, were tested to evaluate the effects of clopidogrel. *In vitro* clopidogrel treatment decreased the percentage of influenza-infected Calu-3 cells, and pretreatment with clopidogrel reduced viral replication [65]. These results suggest that inhibition of influenza replication is linked to the inhibition of the $P2Y_{12}$ signaling pathway by

clopidogrel. However, the clopidogrel prodrug is not readily metabolized *in vitro*, and therefore it is unclear whether the effects observed were due to $P2Y_{12}$ inhibition or an alternative mechanism.

Ras/Raf/MEK/ERK is also a dominant cancer signaling pathway [66]. Mutations of Ras lead to constitutively active Ras proteins, thereby preventing apoptosis. Since apoptosis regulation has been an attractive chemotherapeutic treatment target, chemical inhibitors of this signaling pathway have been considered as potential treatment candidates for many types of cancers [67]. Breast cancer cells have been demonstrated to cause direct and indirect activation of platelets by ADP, thromboxane A2, and metalloproteinases [68–70]. Activated platelets release metalloproteinases, which degrade the vascular basement membrane resulting in tumor growth and metastasis [71]. As a result, clopidogrel was hypothesized to possess anticancer properties. In an experimental model of mammary cancer (transplant of mouse mammary adenocarcinoma 4T1 cells into recipient mice), clopidogrel alone did not have significant anti-tumor activity. However, the anti-tumor effects of 5-fluorouracil, cyclophosphamide, and mitoxantrone were potentiated by concomitant clopidogrel treatment [72]. The protective effects were observed by both a decrease in invasive tumor cells and a decrease in the accumulation of platelets within the tumors. In addition, clopidogrel administered with a nitric oxide donor effectively inhibits metastasis by normalizing endothelial function [73]. Conversely, clopidogrel decreased the efficacy of doxorubicin, cisplatin, and tamoxifen [72]. These agents are all CYP3A4 substrates, and therefore clopidogrel may affect the metabolism of these chemotherapeutics to reduce their efficacy.

Interestingly, clopidogrel may also have utility as a cancer preventative agent. In a prospective trial investigating cancer prevention, patients receiving clopidogrel were monitored for cancer development. Clopidogrel use was associated with a decreased incidence of all cancers, including colorectal cancer, which was reduced by 20-30% [74,75]. Together with the anticancer effects outlined above, these results suggest that clopidogrel may be a beneficial adjunctive agent to existing cancer therapeutic strategies.

3.1.5 Regulation of bone homeostasis and bone marrow function

The $P2Y_{12}$ receptor is suggested to be expressed in the bone and bone marrow of rats and mice [34,39]. Prolonged exposure to clopidogrel has effects on bone mass and bone cell function. Clopidogrel treatment decreased osteoblast number by 50% and reduced cell viability. Bone formation, bone marrow density, and collagen production also decreased after clopidogrel treatment [76]. Interestingly, adipogenic transcription factor levels increased 4.4-fold, and adipocytes increased by 60% with clopidogrel treatment. The reduction in osteoblast number is likely due to clopidogrel's action on precursor cells causing them to follow an adipogenic differentiation pathway rather than the osteoblastic pathway, leading to an increase in adipocytes [76]. Since osteoblasts express the $P2Y_{12}$ receptor, these effects result from clopidogrel-mediated receptor inhibition, but this has not yet been confirmed. Contrasting to these previous studies, however, clopidogrel also enhanced new bone formation in rabbits and mice [77,78]. These results highlight how differences in dosage, treatment duration, and species play a crucial role in the effects of clopidogrel.

3.2 Non-$P2Y_{12}$ Effects of Clopidogrel

Metabolites of clopidogrel are primarily considered "inactive" simply owing to their lack of inhibition of platelet $P2Y_{12}$, the target of M13. However, many of the other metabolites are structurally similar to M13. In addition, members of the P2Y receptor family share a high degree of similarity in both sequence and structure, while their expression has been demonstrated in a diverse number of cell types and tissues. Therefore, simply assuming that clopidogrel metabolites are biologically "inactive" due to a lack of platelet inhibition is short-sighted. There may be, in fact, clopidogrel metabolites with understudied but unique pharmacology. Furthermore, several clopidogrel metabolites are electrophilic species, increasing the likelihood that they may unselectively interact with macromolecules like DNA or proteins, leading to toxic effects.

3.2.1 Regulation of hematopoiesis

In human patients, clopidogrel treatment reduced the white blood cell (WBC) count. A month after clopidogrel was discontinued, the WBC count increased. Interestingly, ticagrelor treatment (a structurally distinct reversible $P2Y_{12}$ antagonist) did not alter the WBC count. However, when patients who received ticagrelor were transitioned to clopidogrel treatment, a reduction in the WBC count was then observed [79]. No change in the WBC count with ticagrelor indicates the mechanism to which clopidogrel decreases the WBC count is $P2Y_{12}$-independent. Future studies are required to determine the mechanism underlying clopidogrel's effect on circulating WBCs.

3.2.2 Inhibition of inflammation

Lipopolysaccharide (LPS) is frequently used to induce experimental inflammation in animals. LPS activates multiple intracellular signaling pathways and transcription factors, including NF-κB [80]. Active NF-κB increases inflammatory cytokines, chemokines, and adhesion molecules while regulating cell proliferation, differentiation, and apoptosis [81]. Clopidogrel inhibits the degradation of IKBα and the phosphorylation of p65, which suppresses NF-κB signaling, thereby reducing inflammatory cytokines, and preventing apoptosis [82]. When clopidogrel was administered to LPS-treated rats, inflammatory lung and liver injuries were reduced. Clopidogrel also reduced the pro-inflammatory cytokine levels in these animals [83]. To determine if these effects were a consequence of $P2Y_{12}$ inhibition, LPS was administered to $P2Y_{12}^{-/-}$ mice. TNF-α, IFN-γ, IL-10, IL-6, IL-4, and keratinocyte-derived chemokine cytokine levels were higher in $P2Y_{12}^{-/-}$ LPS-treated mice than in wild-type LPS-treated mice suggesting that $P2Y_{12}$ is protective in this model of inflammation. Interestingly, $P2Y_{12}^{-/-}$ LPS-treated mice administered clopidogrel exhibited a decrease in inflammation. These results indicate that clopidogrel has $P2Y_{12}$-independent effects capable of reducing inflammation [84]. The effects were also confirmed in a human model of LPS-induced inflammation [85]. Clopidogrel

treatment reduced IL-6, TNF-α, and CCL2 in LPS-treated human volunteers. Furthermore, patients undergoing primary percutaneous coronary intervention who were on clopidogrel avoided increases in high sensitivity C-reactive protein, a marker of systemic inflammation [86]. This beneficial effect of clopidogrel provides evidence for the use of clopidogrel in the treatment of inflammatory diseases.

3.2.3 Changes in vascular function

$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13,}$ and $P2Y_{14}$ receptors are expressed on the vascular smooth muscle and endothelium [38,87,88]. The relative expression of P2Y receptors varies among vascular beds, and therefore the actions of purinergic modulators differ depending on the vessel assessed. Clopidogrel affects the vasculature, both acutely and chronically [54–56,60,89–94]. Acute responses relate to the modulation of vascular function, including the induction of vasodilation and the inhibition of vasoconstriction. Chronic responses involve changes in vessel structure, including regulation of vascular remodeling and the inhibition of angiogenesis.

Clopidogrel induces acute vascular changes without hepatic metabolism [90,92]. Clopidogrel was administered to Langendorff-prepared isolated guinea pig hearts that were perfused retrogradely through the aorta. An ultrasonic flowmeter monitored coronary flow. Clopidogrel increased coronary flow in a concentration-dependent manner, mediated by endothelium-derived nitric oxide [90]. Interestingly, ATP-mediated coronary vasodilation is caused by the activation of A_{2A} receptors rather than P2Y receptors [95]. This suggests clopidogrel is activating A_{2A} receptors rather than P2Y receptors, however, this needs to be examined further. Clopidogrel treatment in isolated caudal arteries from rats showed a concentration-dependent increase in vasodilation in these vessels [92]. However, in contradiction to the previous study with coronary arteries, this finding was not related to endothelium-derived nitric

oxide. The opposing results might be explained by the differences in the specific physiology of each vessel.

Additional studies evaluated tail arteries isolated from clopidogrel-treated rats. Perfusion pressure was measured after adding 2-(methylthio)adenosine 5'-diphosphate (2MeSADP), an analog of ADP that activates the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors. Clopidogrel treatment did not impair constriction in response to 2MeSADP [91]. Furthermore, the aorta from clopidogrel-treated mice was analyzed for contraction to 2MeSADP, and again clopidogrel did not affect 2MeSADP contraction [89]. These data strongly suggest that clopidogrel does not inhibit $P2Y_1$-, $P2Y_{12}$-, and $P2Y_{13}$-mediated contraction.

3.2.4 Changes in vascular structure

Changes in the artery structure are commonly associated with interventional vascular procedures. Intimal hyperplasia is a complication from stent placement, endarterectomy, and vascular reconstruction procedures. To evaluate preventative therapeutics, rats were subjected to carotid endarterectomy. Coadministration of clopidogrel and pravastatin, a hydroxymethylglutaryl Coenzyme-A reductase inhibitor used to treat dyslipidemia, significantly decreased intimal hyperplasia and serum cholesterol levels. Pravastatin treatment alone did not reduce intimal hyperplasia [94]. Interestingly, clopidogrel alone also did not decrease intimal hyperplasia [93]. Further studies are required to identify the synergistic mechanism underlying the simultaneous administration of pravastatin and clopidogrel. It remains to be determined whether this treatment strategy could be a valuable therapy for reducing intimal hyperplasia in a clinical setting.

3.2.5 Clopidogrel-associated bleeding cannot be explained by platelet inhibition alone

Clopidogrel is associated with adverse bleeding, particularly cerebral microbleeds and intracerebral hemorrhages [96–100]. For instance, 30-40% of patients who have received clopidogrel for at least one year had cerebral microbleeds, and patients who received clopidogrel for more than five years have an increased risk of not only cerebral microbleeds but also macroscopic bleeding [99–102]. Cerebral microbleeds increase the likelihood of recurrent intracerebral hemorrhage [103]. This is a significant public health concern because DAPT increases the risk of intracerebral hemorrhage by 42% [104].

Most attribute the adverse bleeding observed with clopidogrel to the antiplatelet properties of the drug. However, several groups have recently discovered that this is not the case. Selatogrel is a reversible antagonist of the $P2Y_{12}$ receptor that produces comparable antithrombotic effects to clopidogrel, albeit with a wider therapeutic window. Crescence and colleagues compared tail blood loss and bleeding time in selatogrel- and clopidogrel-treated mice. Their results revealed that bleeding time in clopidogrel-treated animals was more than 8-fold longer than in selatogrel-treated animals. Additionally, clopidogrel treatment increased blood loss 34-fold, while selatogrel treatment only induced a 4-fold increase in blood loss [105]. To further characterize the effect(s) of selatogrel treatment, calcium mobilization was quantified in the endothelial cell layer from cremaster muscle arterioles after damage by laser injury. Calcium mobilization was unchanged in $P2Y_{12}^{-/-}$ mice compared to wild-type mice indicating calcium mobilization is a $P2Y_{12}$-independent mechanism. Selatogrel did not alter calcium mobilization in $P2Y_{12}^{-/-}$ mice compared to vehicle treatment, concluding that selatogrel was a highly selective $P2Y_{12}$ antagonist devoid of off-target effects [105]. Unfortunately, the inhibitory effects of clopidogrel on calcium release in $P2Y_{12}^{-/-}$ mice were not determined.

Subsequently, André and colleagues evaluated clopidogrel treatment in $P2Y_{12}^{-/-}$ mice and found a significant increase in blood loss compared to vehicle-treated $P2Y_{12}^{-/-}$ mice [106]. This study represented one of the first reports of the potentiation of bleeding by clopidogrel in $P2Y_{12}^{-}$

$^{/-}$ animals and strongly suggests that the bleeding effects associated with this drug are mediated, in part, by $P2Y_{12}$-independent effects.

The key to appreciating these $P2Y_{12}$-independent effects is likely a complete map of the structure and pharmacology of clopidogrel metabolites. The M15 metabolite was the first to be assessed for non-platelet, non-$P2Y_{12}$ effects in the body. The M15 endo metabolite of clopidogrel undergoes spontaneous hydrolysis to release hydrogen sulfide (H_2S) [14]. H_2S is an important regulator of the cardiovascular system and mediates intracellular signal transduction, much like nitric oxide or carbon dioxide. It regulates the cell cycle, apoptosis, and oxidative stress. H_2S donors reduce thrombus formation and occlusion [109]. To test the ability of the M15 metabolite to minimize thrombus formation, $FeCl_3$-mediated carotid artery injury was induced in mice, and time to occlusion was recorded. The M15 metabolite was shown to prolong the time to occlusion in mice significantly. This result provides evidence that a previously classified “inactive” metabolite, M15, may be pharmacologically active through the release of H_2S, thereby interfering with hemostasis [14]. These findings suggest that while specific clopidogrel metabolites have not been evaluated for off-target bleeding, M15 represents an exciting candidate that might be responsible, in part, for these adverse effects. The understudied metabolites of clopidogrel must be further analyzed to evaluate this possibility.

The first step in elucidating the adverse effects of clopidogrel was to distinguish the effects of the active metabolite, M13. Our group has developed a conjugate of M13, termed DT-678. DT-678 produces M13 through bioactivation with glutathione. I hypothesized DT-678 and clopidogrel were equally effective at inhibiting platelets, with DT-678 having reduced adverse bleeding. We postulated DT-678 would not have adverse side effects since the additional 15+ metabolites of clopidogrel were not produced. The next part of this chapter will compare the antiplatelet properties and bleeding tendencies of DT-678, clopidogrel, and ticagrelor to determine if DT-678 is a cleaner antiplatelet agent.

Part 2

DT-678 Inhibits Platelet Activation with a Lower Tendency for Bleeding Compared to Existing $P2Y_{12}$ Antagonists

Modified from: Kuszynski DS, Lauver DA, Christian BD, Bernard MP, Teuber JP, Markham BE, Chen YE, Zhang H. DT-678 inhibits platelet activation with lower tendency for bleeding compared to existing $P2Y_{12}$ antagonists. Pharmacol Res Perspect. 2019 Jul 25;7(4):e00509. doi: 10.1002/prp2.509. PMID: 31372229; PMCID: PMC6658415.

Abstract

The novel clopidogrel conjugate, DT-678, is an effective inhibitor of platelets and thrombosis in preclinical studies. However, a comparison of the bleeding risk with DT-678 and currently approved $P2Y_{12}$ antagonists have yet to be determined. The objective of this study was to evaluate the bleeding tendency of animals treated with clopidogrel, ticagrelor, and DT-678. Ninety-one New Zealand white rabbits were randomized to one of 13 treatment groups (n = 7). Platelet activation was assessed by flow cytometry and light transmission aggregometry before and after the administration of various doses of DT-678, clopidogrel, and ticagrelor. Tongue template bleeding times were also measured before and after drug treatment. Treatment with $P2Y_{12}$ receptor antagonists caused a dose-dependent reduction in markers of platelet activation (P-selectin and integrin $\alpha_{IIb}\beta_3$) and aggregation in response to adenosine diphosphate stimulation. At the same doses required for platelet inhibition, clopidogrel and ticagrelor significantly prolonged bleeding times, while DT-678 did not. DT-678 and the FDA-approved $P2Y_{12}$ antagonists, clopidogrel and ticagrelor, are effective inhibitors of platelet activation and aggregation. However, unlike clopidogrel and ticagrelor, DT-678 did not prolong bleeding times at equally effective antiplatelet doses. The results suggest a more favorable benefit/risk ratio for DT-678 and potential utility as part of a dual antiplatelet therapy regimen.

1. Introduction

Together with aspirin, purinergic $P2Y_{12}$ receptor antagonists, like clopidogrel and ticagrelor, are widely used in dual antiplatelet therapy (DAPT) for the prevention of thrombosis in patients with acute coronary syndrome (ACS) [110-114]. Approximately, one million patients receive DAPT for ACS in the United States every year. Recent clinical trials have demonstrated the benefits of DAPT beyond 1 year and it is anticipated that long-term use of DAPT will steadily increase [115,116]. The most concerning adverse event associated with any antithrombotic therapy is bleeding. Head-to-head comparison of bleeding tendency between $P2Y_{12}$ antagonists is difficult since the classification of severity and clinical relevance of bleeding events differ in many large clinical trials. Additionally, even minor bleeding events, while not life-threatening in and of themselves, are significant since they are one of the most important reasons for antiplatelet therapy nonadherence which can leave patients at increased risk for thrombotic events [117]. Despite the approval of newer, more efficacious agents, clopidogrel continues to be broadly used in clinical cardiology. The comparative bleeding safety of clopidogrel compared to the newer agents like prasugrel and ticagrelor has been demonstrated in multiple large-scale clinical trials [98,118]. In the Platelet Inhibition and Patient Outcomes (PLATO) trial, ticagrelor significantly increased spontaneous bleeds, major bleeds, major plus minor bleeds, and major plus minor plus minimal bleeds compared to clopidogrel. Therefore, clopidogrel is the preferred agent for long-term management of patients. Clopidogrel, however, is subject to several limitations which include interpatient variability, delayed onset of action, and frequent drug-drug interactions [119,120]. In addition, approximately 30% of Caucasians and 60%-70% of Asians fail to respond to clopidogrel therapy due to polymorphisms in CYP2C19 [119,121,122]. As a result, these patients have an increased risk of major adverse cardiovascular events [122,123].

Our group has previously reported the development of DT-678 (née ClopNPT), a disulfide conjugate of the clopidogrel metabolite, M13, with 3-nitropyridine-2-thiol [107,108,124]. In the presence of glutathione, DT-678 is readily converted to M13 through a disulfide exchange

reaction as illustrated in Figure 3 [107]. Our earlier studies have demonstrated significant inhibition of *ex vivo* platelet aggregation and thrombosis by DT-678 in mice and rabbits [108,124]. Furthermore, we have established that DT-678 releases the M13 with a Tmax of 1000 ng/mL after a 5 mg/kg intravenous dose or a 10 mg/kg oral dose in mice [108]. These results suggest that DT-678 has favorable pharmacokinetic/pharmacodynamic properties that may potentially overcome the attenuated pharmacokinetic properties of clopidogrel and thus significantly improve the efficacy of antiplatelet therapy.

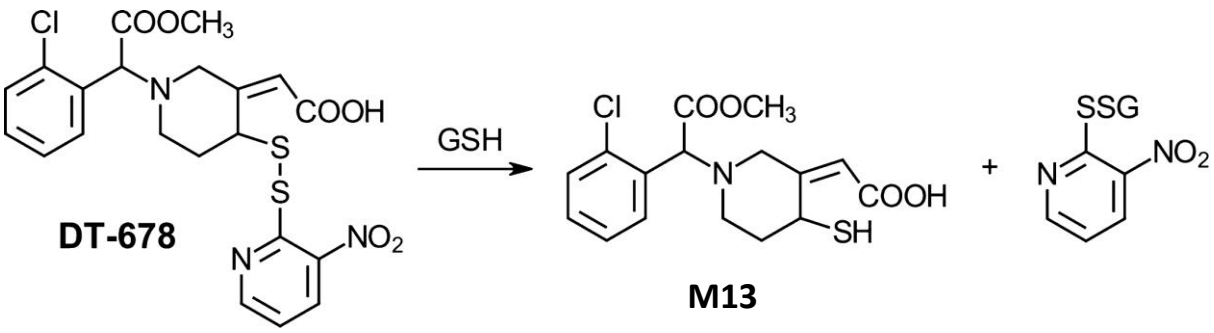

Figure 3: DT-678 bioactivation. In the presence of glutathione (GSH), DT-678 releases the clopidogrel active metabolite (M13) via a thiol exchange reaction without the requirement of CYP2C19.

In this study, I sought to further evaluate the compound by comparing antiplatelet activity and bleeding tendency in animals treated with the approved $P2Y_{12}$ antagonists, clopidogrel and ticagrelor, or DT-678. Our results demonstrate dose-dependent inhibition of platelet aggregation and activation by all agents. However, bleeding times were significantly prolonged by clopidogrel and ticagrelor, but not DT-678. These findings suggest that DT-678 may be safer in the clinical setting while maintaining similar antiplatelet efficacy.

2. Materials and Methods

2.1 Chemicals

Racemic DT-678 was synthesized and purified to 99% by Beijing SL Pharmaceuticals. S-clopidogrel and ticagrelor were purchased from Cayman Chemical Co. Alexa Fluor 647-tagged anti-CD62P antibody was purchased from MBL (D280-A64) and fibrinogen-FITC (fluorescein isothiocyanate) was purchased from Abcam (ab92788). Adenosine diphosphate (ADP), arachidonic acid (AA), and collagen were purchased from Chrono-log Corporation. Polyethylene glycol 400 and N, N-dimethylacetamide (DMA) were purchased from Millipore Sigma.

2.2 Animal Care and Use

The procedures used in this study were in accordance with the Michigan State University guidelines and were approved by the Institutional Animal Care and Use Committee (Animal Use Form 07/17-115). Michigan State University Campus Animal Resources provided all veterinary care.

2.3 Surgical Preparation of Rabbits and Administration of Drugs

Ninety-one male New Zealand white rabbits (1.9-2.4 kg) were obtained from Charles River Laboratories, Inc (Wilmington, MA). All animals were acclimated for a minimum of 5 days and had free access to standard chow and fresh water before the study. Animals were maintained on an automated 12/12-hour light/dark cycle with 7:00 am as the start of the light phase. On the day of the study, rabbits were sedated and anesthetized to surgical unconsciousness with ketamine (40 mg/kg, intramuscular [IM]), xylazine (5 mg/kg, IM), and isoflurane (1%-3%, inhaled). The surgical site was shaved, and the rabbits were placed on a 37°C heating pad. Isoflurane was administered through a mask that was placed over the mouth and nose. The

right jugular vein was surgically isolated and cannulated with a polyethylene cannula for drug administration and blood collection. Respiratory rate, the lead II electrocardiogram, heart rate, and body temperature were monitored throughout the procedure. Vehicle, DT-678 (0.1-3.0 mg/kg), clopidogrel (0.3-10.0 mg/kg), or ticagrelor (0.1-3.0 mg/kg) were administered via the jugular cannula (n = 7 per dose group). $P2Y_{12}$ antagonists were dissolved in a 20:80 (v/v) mixture of DMA and polyethylene glycol 400. Drugs were administered as an intravenous bolus injection at the indicated doses.

2.4 Collection of Whole Blood

Blood samples were collected from the jugular cannula into a syringe containing 3.2% sodium citrate as an anticoagulant (1:10 citrate to blood ratio) before (baseline) and 10 minutes, 1 hour, and 2 hours after drug treatment. The blood samples were divided into two parts: 1.5 mL was used to perform flow cytometry (baseline and 1 hour posttreatment time points only) while the remainder was used for platelet aggregometry (see below).

2.5 Determination of Platelet Activation by Flow Cytometry

Platelet activation was determined by anti-CD62P-alexa fluor 647 and fibrinogen-FITC binding in whole blood stimulated by ADP. Citrated blood (450 µL) was incubated with ADP (20 µmol/L) or HEPES-buffered saline for 2 minutes. Fibrinogen-FITC (0.17 mg/mL) was then added to these samples and incubated for 15 minutes in the dark. The blood was fixed with 1 mL of 1% paraformaldehyde for 15 minutes to prevent aggregation and washed with 1 mL Dulbecco's phosphate-buffered saline (DPBS). Subsequently, an anti-CD62P antibody (0.5 µg/mL) was added to the samples and incubated for 15 minutes followed by washing and resuspension in DPBS. Flow cytometric assessment was performed using a BD Accuri C6 (BD Biosciences) available in the MSU South Campus Flow Cytometry Core Facility. Events (20,000) were collected on a log scale for FSC-A and SSC-A, gated on the platelet scatter-

based population, followed by doublet discrimination. Quadrant gates for fibrinogen-FITC and anti-CD62P-alexa fluor 647 positive events were generated based on fluorescence minus one control prepared for each animal and time point. Double-positive ($CD62P^+$ $fibrinogen^+$) platelets were quantified as a measure of platelet activation. Data were analyzed using CFlow Plus software, v1.0.227.04 (BD Biosciences).

2.6 Determination of Platelet Aggregation by Light Transmission Aggregometry

Platelet reactivity was determined in platelet-rich plasma (PRP) obtained from whole blood samples using light transmission aggregometry. Whole blood samples (see above) were centrifuged at 150 g for 10 minutes at room temperature and the supernatant was collected. The pellet was then centrifuged at 1500 g at room temperature for 10 minutes to obtain the platelet-poor plasma (PPP). Ex vivo platelet aggregation was assessed using a 4-channel aggregometer (Chrono-log Corporation Model 700; Chrono-log Corporation). PRP was continually stirred and maintained at 37°C during the assay. The change in light transmission relative to PPP after stimulation with platelet agonists (ADP [20 µmol/L], AA [500 µmol/L], and collagen [2 µg/ mL]) was recorded.

2.7 Determination of Bleeding Time in New Zealand White Rabbits

To evaluate the bleeding risk of the $P2Y_{12}$ antagonists, bleeding times were measured using a Surgicutt® device (Accriva Diagnostics), which creates a uniform 5-mm long and 1-mm deep incision on the upper surface of the tongue. The margins of the lesion were blotted every 10 seconds with filter paper until blood was no longer transferred from the tongue to the filter paper. The interval from the time the incision was created to the time that blood was no longer apparent on the filter paper is considered the tongue bleeding time. Bleeding times were assessed before treatment and 2 hours after treatment.

2.8 Statistical Analysis

Data were analyzed using GraphPad Prism 7 software (GraphPad Software) and are presented as mean ± SEM. Statistical differences between vehicle and drug treatment groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. Results were considered significant at $p<0.05$. Significance was denoted as $*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$.

3. Results

3.1 $P2Y_{12}$ Antagonists Decrease α-granule Secretion and the Formation of Integrin $\alpha_{IIb}\beta_3$

The effects of $P2Y_{12}$ antagonist treatment on α-granule secretion and the formation of integrin $\alpha_{IIb}\beta_3$ in rabbit platelets were measured by flow cytometry. α-granule secretion was determined by measurement of P-selectin (CD62P) expression on the platelet surface. Integrin $\alpha_{IIb}\beta_3$ expression was measured by the relative binding of fibrinogen-FITC. Treatment with DT-678, clopidogrel, and ticagrelor dose-dependently decreased both α-granule secretion and the formation of integrin $\alpha_{IIb}\beta_3$ on platelets in response to ADP activation compared to vehicle (Figure 4). *Ex vivo* activation of platelets from vehicle-treated animals resulted in 33.36 ± 5.49% double-positive cells ($CD62^+$ $fibrinogen^+$), while double-positive platelets from animals with the highest doses of antagonists were significantly lower (5.96 ± 1.31%, 7.38 ± 1.88%, and 9.82 ± 1.41% for DT-678, clopidogrel, and ticagrelor, respectively).

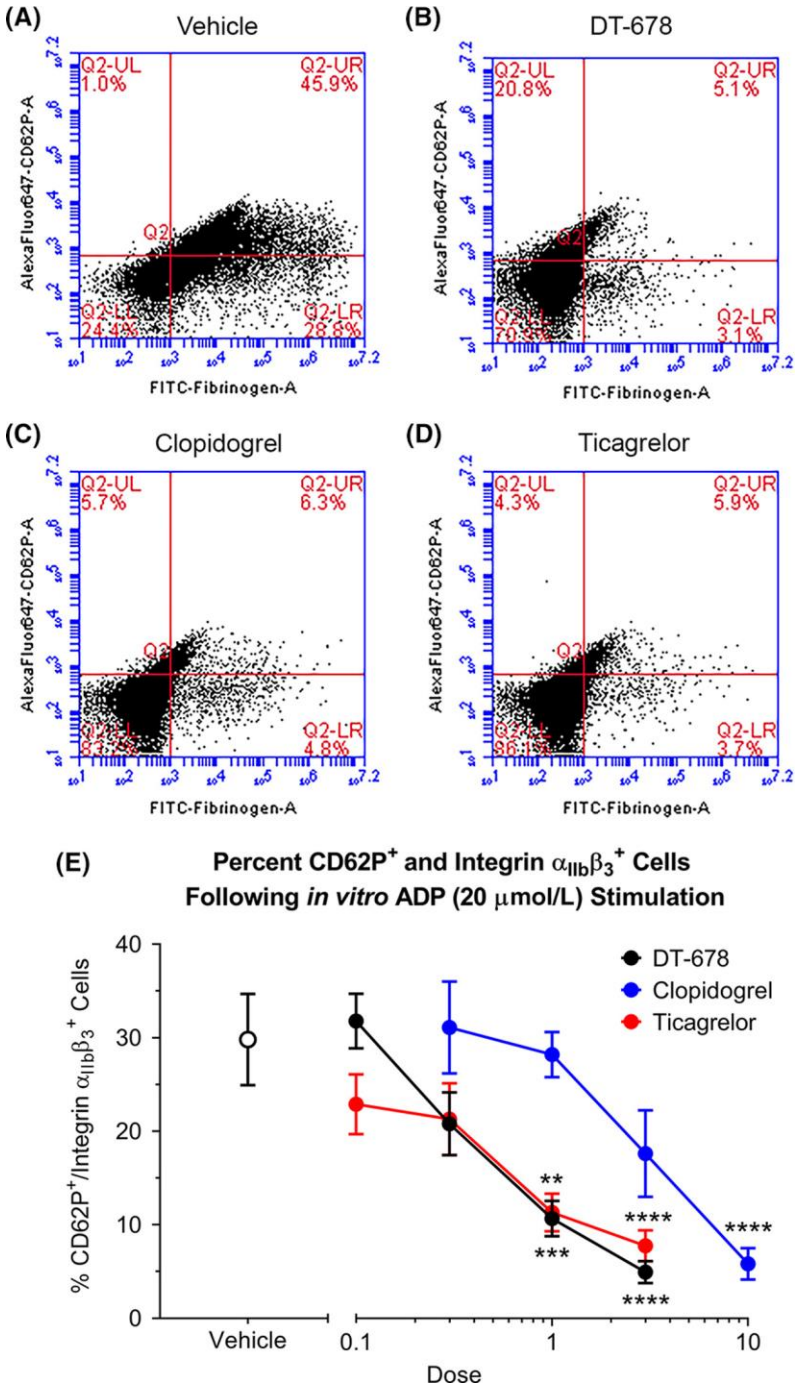

Figure 4: Platelet activation after DT-678, clopidogrel, and ticagrelor treatment in rabbits. Flow cytometric assessment of platelets activated with ADP. Representative scatter plots of platelets from animals treated with (A) vehicle, (B) 3.0 mg/kg DT-678, (C) 10.0 mg/kg clopidogrel, and (D) 3.0 mg/kg ticagrelor. (E) Pretreatment with antiplatelet agents caused a dose-dependent reduction in α-granule secretion (as measured by CD62P expression) and the formation of integrin $\alpha_{IIb}\beta_3$ (indicated by FITC-fibrinogen binding) in response to ADP activation. Double-positive ($CD62P^+$fibrinogen$^+$) events were quantified in the upper right quadrant of individual animal flow cytometric dots plots. The data are presented as the mean ± SEM of seven separate experiments. ***p*<0.01, ****p*<0.001, *****p*<0.0001 when compared with the vehicle-treated group by one-way ANOVA followed by Dunnett's post hoc test.

3.2 Ex Vivo Platelet Aggregation is Dose-dependently Inhibited by $P2Y_{12}$ Antagonist Treatment

Ex vivo aggregation of platelets was measured by light transmission aggregometry using platelets isolated from blood drawn from rabbits treated with different concentrations of DT-678, clopidogrel, or ticagrelor. ADP (20 µmol/L)-induced platelet aggregation was dose-dependently inhibited by treatment with DT-678 (3 mg/kg, 27.2 ± 6.4%), clopidogrel (10 mg/kg, 34.4 ± 5.9%), and ticagrelor (3 mg/kg, 41.6 ± 2.7%) compared to vehicle (83.6 ± 3.5%; Figure 5A). However, AA- (500 µmol/L) and collagen- (2 µg/mL) induced aggregations were relatively unaffected (Figure 5B, 5C, respectively). While maximum inhibition of ADP-induced aggregation was observed 2 hours after the administration of drugs, similar results were recorded at 10 minutes and 1 hour (Figure 6). No change in AA- or collagen-induced aggregation was detected at any time point.

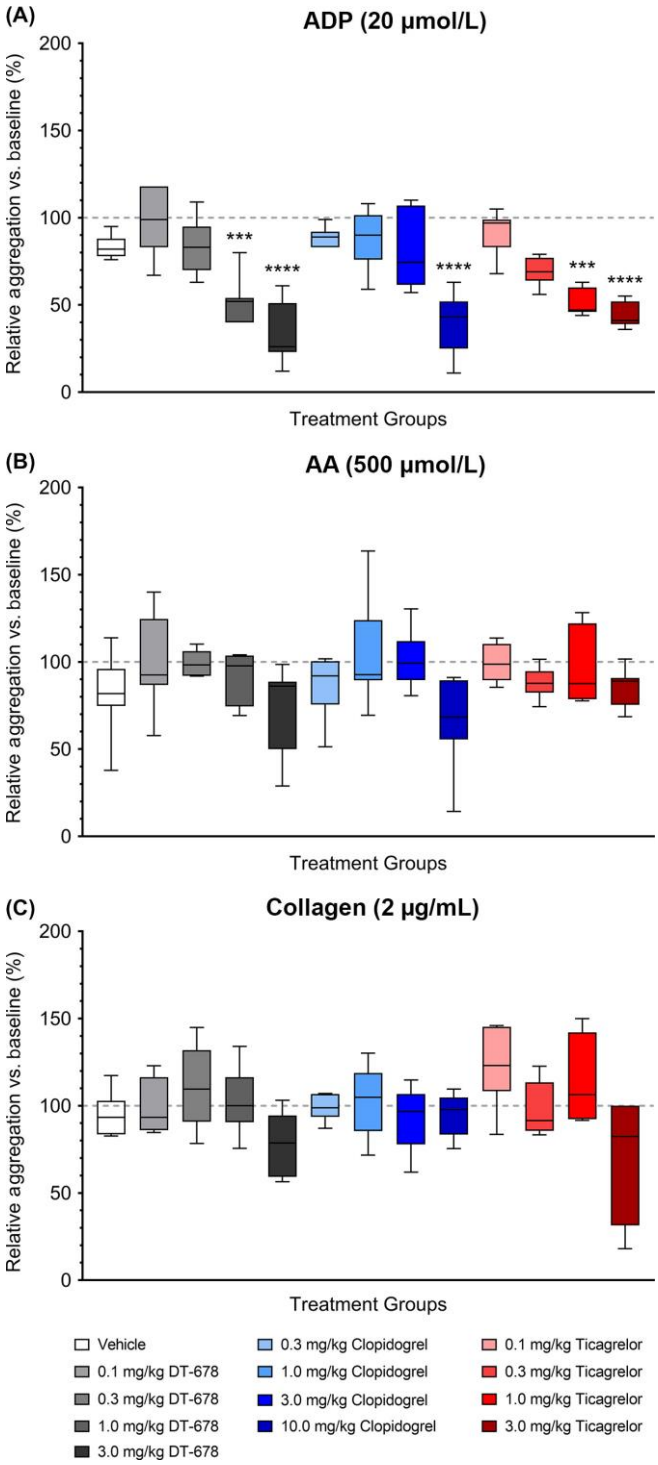

Figure 5: Platelet aggregation after DT-678, clopidogrel, and ticagrelor treatment in rabbits at 2 hours. Percent platelet aggregation responses to (A) ADP (20 µmol/L), (B) AA (500 µmol/L), and (C) collagen (2 µg/mL) for animals treated with vehicle, DT-678 (0.1, 0.3, 1.0, or 3.0 mg/kg), clopidogrel (0.3, 1.0, 3.0, 10.0 mg/kg), or ticagrelor (0.1, 0.3, 1.0, or 3.0 mg/kg). Blood was collected before and 2 h after the administration of drugs. The data are presented as a box and whisker plot of percent change relative to baseline and represent data from five to seven separate experiments. The middle line indicates the median and the lower and upper bars represent the minimum and maximum values, respectively. The box extends from the 25th to the 75th percentiles. ***p<0.001, ****p<0.0001 when compared with the vehicle-treated group by one-way ANOVA followed by Dunnett's post hoc test.

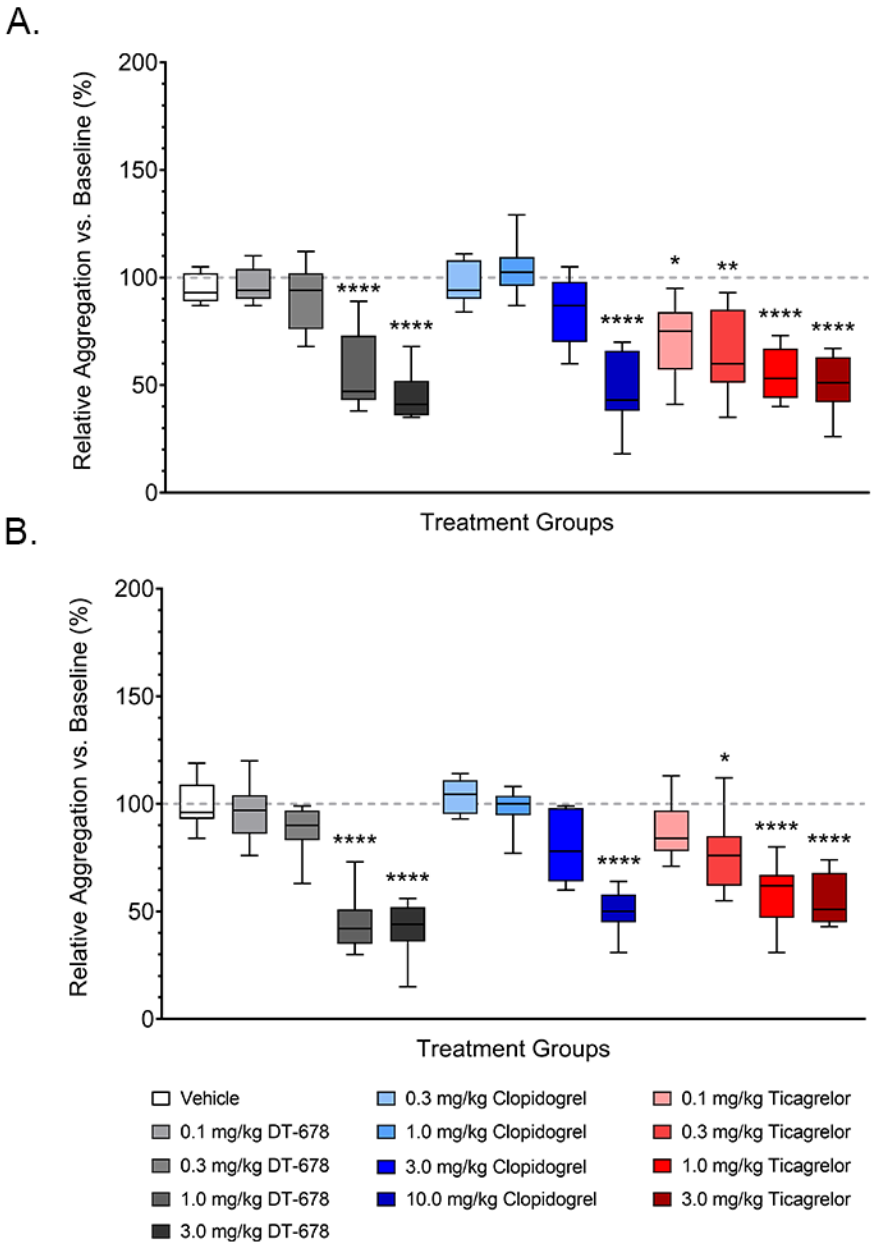

Figure 6: ADP platelet aggregation after DT-678, clopidogrel, and ticagrelor treatment in rabbits at 10 minutes and 1 hour. Percent platelet aggregation responses to ADP (20 µM) for animals treated with vehicle, DT-678 (0.1, 0.3, 1.0 or 3.0 mg/kg), clopidogrel (0.3, 1.0, 3.0, 10.0 mg/kg) or ticagrelor (0.1, 0.3, 1.0 or 3.0 mg/kg). Blood was collected before, and 10 minutes or 1 hour after the administration of drugs. The data are presented as a box and whisker plot of percent change relative to baseline (before treatment) for (A) 10 minutes and (B) 1 hour after the administration of drugs. The values represent data from 7 separate experiments. The middle line indicates the median and the lower and upper bars represent the minimum and maximum values, respectively. The box extends from the 25th to the 75th percentiles. *p<0.05, **p<0.01, ****p<0.0001 when compared with the vehicle-treated group by one-way ANOVA followed by Dunnett's post hoc test.

3.3 Tongue Bleeding Time is Significantly Prolonged by Treatment with Clopidogrel and Ticagrelor, but not DT-678

Tongue template bleeding time was assessed using a Surgicutt® device. Bleeding times were similar at baseline in all the treatment groups (data not shown). Treatment with antiplatelet doses of clopidogrel (3.0 and 10.0 mg/kg; 231.4 ± 42.8% and 235.4 ± 38.2%, respectively) and ticagrelor (1.0 and 3.0 mg/kg; 216.0 ± 53.0% and 265.6 ± 23.9%, respectively) significantly prolonged bleeding times 2 hours after treatment compared to vehicle (92.2 ± 9.2%; Figure 7). Treatment with antiplatelet doses of DT-678 (1.0 and 3.0 mg/ kg; 155.6 ± 28.1% and 172.2 ± 17.0%, respectively) modestly prolonged bleeding time, but the difference was not statistically significant. The two lowest doses of all drugs did not significantly increase the bleeding time (Figure 8).

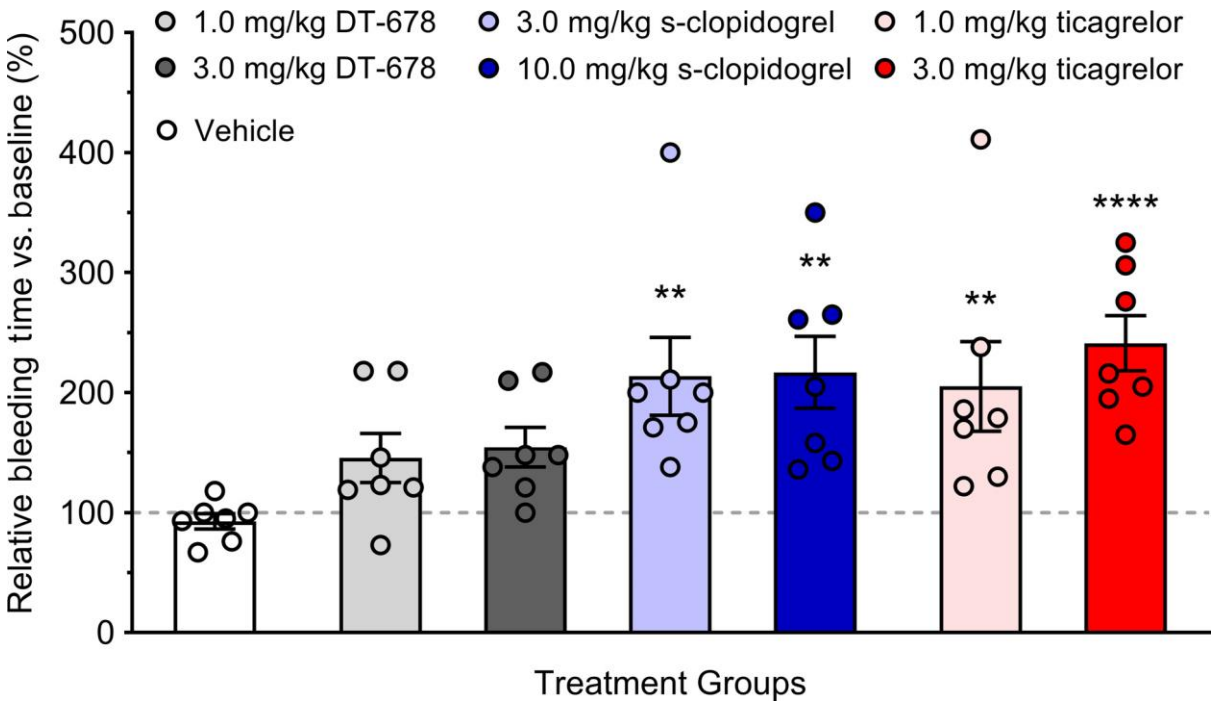

Figure 7: Bleeding time after DT-678, clopidogrel, and ticagrelor treatment in rabbits at 2 hours. Tongue bleeding time after treatment with vehicle, DT-678 (1.0 or 3.0 mg/kg), clopidogrel (3.0 or 10.0 mg/kg), or ticagrelor (1.0 or 3.0 mg/kg). Bleeding times were assessed at baseline and 2 h after the administration of drugs. The data are presented as percent change relative to baseline and represent the mean of seven experiments ± SEM. ***p*<0.01, *****p*<0.0001 when compared with the vehicle-treated group by one-way ANOVA followed by Dunnett's post hoc test.

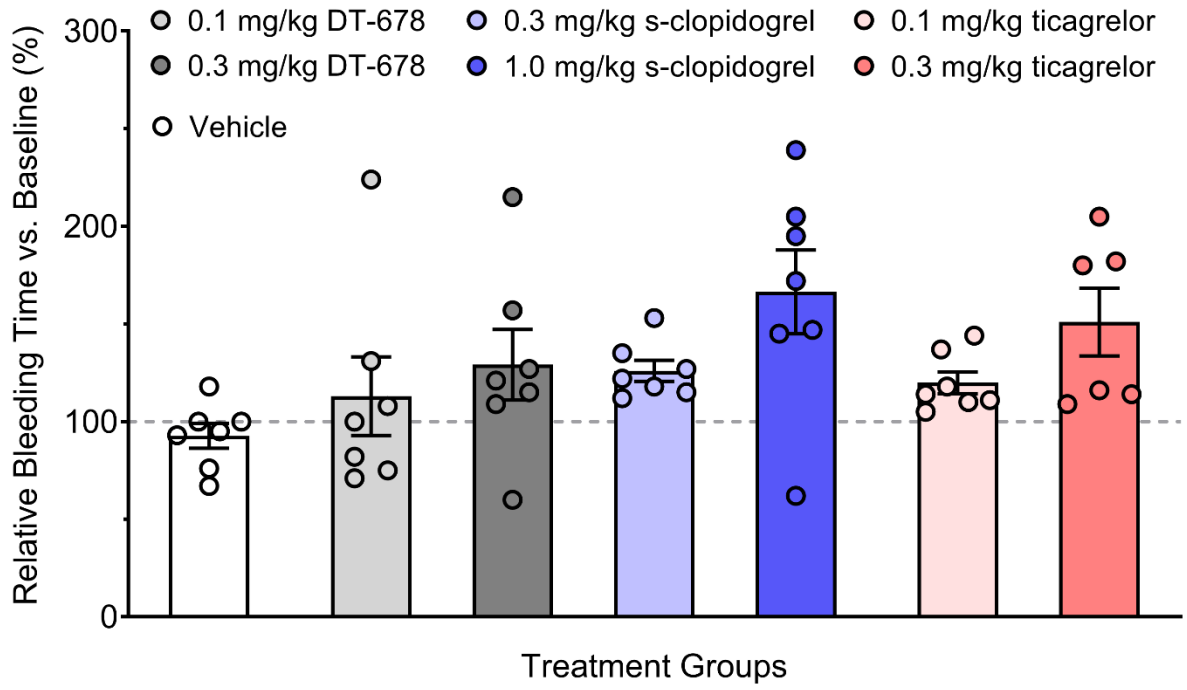

Figure 8: Bleeding time after DT-678, clopidogrel, and ticagrelor treatment in rabbits at low doses at 2 hours. Tongue bleeding time after treatment with vehicle, DT-678 (0.1 or 0.3 mg/kg), clopidogrel (0.3, 1.0 mg/kg), or ticagrelor (0.1 or 0.3 mg/kg). Bleeding times were assessed at baseline and 2 hours after the administration of drugs. The data are presented as percent change relative to baseline and represent the mean of 7 experiments ± SEM.

4. Discussion

Antagonists of $P2Y_{12}$ receptors are an important component in the pharmacological management of patients at risk for thrombotic events. Prescribed together with low-dose aspirin, these agents have been proven effective at reducing the risk of heart attack and stroke. Despite the availability of multiple $P2Y_{12}$ antagonists recommended for DAPT, interpatient variability persists in a significant number of ACS patients, which leads to an increased risk of ischemic complications and reduced survival rate. Although clopidogrel is generally effective and well-tolerated, it has well-documented clinical limitations such as interpatient variability, delayed onset of action, and drug-drug interactions. Approximately, 30% of Caucasians and up to 70% of Asians are resistant to clopidogrel [122,125,126]. Genetic polymorphisms in CYP2C19 are the main contributor to this lack of responsiveness. Patients who carry CYP2C19 loss-of-function mutations fail to effectively metabolize the

clopidogrel prodrug to M13. More recently a reversible $P2Y_{12}$ antagonist, ticagrelor, has been developed. This agent differs from previous thienopyridine agents in that it is not a prodrug and therefore does not require bioactivation. Ticagrelor, however, is subject to CYP3A4-mediated metabolism and its primary metabolite is also a potent $P2Y_{12}$ inhibitor [127]. Ticagrelor is prone to numerous adverse drug interactions due to the induction and inhibition of CYP3A4 by many clinically used drugs including ketoconazole, atazanavir, ritonavir, rifampin, dexamethasone, statins, and more [128]. Although newer agents like ticagrelor have improved clinical outcomes, they also increase the risks of bleeding [129,130]. The primary safety concern with ticagrelor is bleeding as indicated in the PLATO trial supporting the approval of ticagrelor by the FDA [129]. Patients taking ticagrelor are nine times more likely to discontinue the use of the drug than those on clopidogrel.

Due to the numerous limitations with $P2Y_{12}$ antagonists, our research team has developed a novel conjugate of clopidogrel that spontaneously and nonenzymatically releases M13 after oral and intravenous administration [107,108,124]. Our earlier studies have demonstrated the rapid release of M13 within minutes of administration [108]. In this study, we report our findings comparing the antiplatelet and bleeding effects of DT-678 to clopidogrel and ticagrelor. The inhibitory effects of these agents on platelet activation were evident in the reduced surface expression of P-selectin and decreased binding of fluorescently labeled fibrinogen in response to ADP activation. In addition, ADP-induced platelet aggregation was dose-dependently inhibited by treatment with DT-678, clopidogrel, and ticagrelor. Importantly, however, tongue template bleeding times were only significantly prolonged by treatment of clopidogrel and ticagrelor and not DT-678 suggesting that the latter has a more favorable safety profile.

Preclinical assessment of bleeding risk is limited by the availability of standardized animal models. A great deal of effort has been devoted to characterizing the murine tail cut assay in assessing the bleeding tendency of antithrombotic drugs and genetic hemostatic disorders. The severity of the tail amputation, however, does not accurately replicate the clinical state. Furthermore, there is no common protocol for testing and as a result, bleeding times vary

considerably among laboratories. This inconsistency makes direct comparison of the adverse bleeding effects of drugs difficult [131,132]. Template bleeding tests in humans were first described by Milian in 1901 and were later improved by several others [133-135]. The tests involve making a small incision on the skin and recording the time required for blood flow to cease. Due to the global coverage of fur on the body of most laboratory animal species, our laboratory has adapted this assay to use the upper surface of the tongue while the animal is under anesthesia [136,137]. We have routinely used bleeding devices that create a reproducible incision with respect to length and depth allowing for reliable comparisons between time points and animals possible. While this model does not accurately mimic spontaneous bleeding in humans, the severity of the incision more closely reflects clinical bleeding tests.

In the current study, tongue bleeding times were significantly increased in animals treated with FDA-approved $P2Y_{12}$ antagonists, but not DT-678 which possesses the same pharmacological cargo as clopidogrel. The doses of agents used in this study were based on their antiplatelet efficacy which was empirically determined by flow cytometry and platelet aggregation. These results are in partial agreement with our previously reported bleeding data [108]. In that study, no increase in bleeding time was observed with DT-678 at a dose of 1 mg/kg; however, there was a significant increase in bleeding at a dose of 2 mg/kg. At present, we do not have an explanation for the observed differences in bleeding. However, no comparative analysis was performed with other $P2Y_{12}$ antagonists in the previous study and therefore it is unknown whether clopidogrel or ticagrelor would have further prolonged the bleeding time.

There are multiple explanations for the observed bleeding results in the present study. Clopidogrel undergoes a complicated metabolism pathway in which at least 15 different compounds are created in addition to M13. Literature evidence suggests that some of these compounds may possess biological activity. In fact, the M15 metabolite has recently been identified by Zhu and colleagues [14] which possesses thiol-mediated antiplatelet activity

separate from the inhibition of $P2Y_{12}$. The additive effects of these “nonactive” metabolites of clopidogrel may therefore potentiate bleeding in animals and humans.

With respect to ticagrelor, the compound has been reported to have potential off-target effects on purinergic receptors in the vasculature leading to vasodilation [89,91]. Ticagrelor is structurally distinct from the thienopyridine class of antiplatelet agents and as such may possess differential actions at purinergic receptors in distinct tissues. In addition, increased circulating adenosine concentrations have also been reported in patients taking ticagrelor which might also explain some of the vascular effects associated with the drug [138]. These vascular properties of ticagrelor may result in an increased bleeding tendency in the presence of simultaneous inhibition of platelet $P2Y_{12}$. The importance of these effects is uncertain, however, as more recent reports in humans suggest no difference in the vascular effects of thienopyridines and ticagrelor [139].

An interesting observation from our results is that clopidogrel required an approximately 10-fold higher dosage than DT-678 to elicit similar antiplatelet effects. A likely explanation for this finding is that the clopidogrel prodrug undergoes a complicated metabolism pathway in which only 1%-5% of the administered dose is converted to M13 [13,18]. DT-678, on the other hand, is non-enzymatically converted to M13 and therefore all the administered dose is available for inhibition of $P2Y_{12}$. This finding is potentially important in the context of Type II diabetic patients treated with DAPT. This subset of patients has an impaired ability to form M13 [28,29]. The underlying effect is hypothesized to result from dysregulation of CYP450 enzymes [140,141]. CYP450-independent activation of M13 as with DT-678 may find utility in this unique population. It is important to note, however, that in the present study, rabbits were treated with a single intravenous injection of each drug. Further investigation is required to determine whether the observed effects persist with chronic oral administration.

I conclude that in an experimental bleeding model in rabbits, DT-678 did not significantly prolong bleeding time at doses that were capable of inhibiting platelet activation and aggregation. Conversely, administration of clopidogrel or ticagrelor significantly prolonged

bleeding time at equally effective antiplatelet doses. Given its simplified activation pathway and favorable pharmacokinetics, our results suggest that DT-678 is a potentially useful alternative to existing $P2Y_{12}$ antagonists with improved predictability and safety.

Part 3

Overview of Aims

Introduction

There is a critical need to determine the off-target effects of clopidogrel to understand the mechanisms responsible for the adverse bleeding events. Without this knowledge, new antiplatelet therapies with reduced bleeding side effects cannot be developed. A more complete understanding of the cellular pathways affected by clopidogrel, and its metabolites, is required to decrease risk.

My overall goal for this dissertation was to characterize the off-target effects of clopidogrel and determine how those effects promote bleeding in the cerebral vasculature. Cerebral microbleeds and intracerebral hemorrhages can be instigated by dysregulation of cerebral blood flow and pressure (142). Cerebral blood flow regulation is essential in maintaining blood-brain barrier homeostasis (142). Large arteries, like the middle cerebral artery and posterior cerebral artery, regulate cerebral blood flow through the development of myogenic tone (142-147). Myogenic tone protects the capillary bed from changes in pressure that could lead to cerebral microbleeds and intracerebral hemorrhage (142, 145). When pressure increases in cerebral arteries, myogenic tone increases to regulate blood flow (142). In this dissertation, I determined how platelet activation and bleeding time were altered in both rabbits and mice, as well as how myogenic tone generation, vasodilation, and vasoconstriction of the middle cerebral artery and posterior cerebral artery were altered. I further determined the effects of clopidogrel on specific purinergic receptors in P2Y-stably transfected 1321N1 cells. The interaction of the clopidogrel metabolites with additional purinergic receptors expressed in the smooth muscle ($P2Y_2$, $P2Y_4$, and $P2Y_6$) or the endothelium ($P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$) may be responsible for the observed effect. My central hypothesis was that clopidogrel or its metabolites modulate $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and/or $P2Y_{14}$ signaling in the vasculature which results in adverse bleeding.

Specific Aims

Aim 1: Determine the effect of selective purinergic agonists in the middle cerebral artery from clopidogrel-treated rabbits.

The second chapter of this dissertation examined the effects clopidogrel has on the middle cerebral arteries in rabbits. I hypothesized that clopidogrel or its metabolites interact with $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and/or $P2Y_{14}$ in the middle cerebral artery to impair myogenic tone generation and dilation/constriction. These studies elucidated if clopidogrel or its metabolites alter purinergic receptor activation.

Aim 2: Identify the receptor pathways modulated by clopidogrel or its metabolites.

The third chapter of this dissertation determined if the prodrug clopidogrel or its metabolites, M1 and M2, blocked $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and/or $P2Y_{12}$ calcium release in stably transfected 1321N1 astrocytoma cells. Additionally, mice treated with clopidogrel, M1, or M2 were analyzed for changes in bleeding time and platelet activation. I hypothesized that the prodrug clopidogrel or one of its metabolites inhibits $P2Y_1$, $P2Y_2$, $P2Y_4$, and/or $P2Y_6$, as well as $P2Y_{12}$ in stably transfected cells. Furthermore, I hypothesized that M1 increases bleeding time similarly to clopidogrel bleeding time. Therefore, these studies examined the possibility of alternative purinergic receptor inhibition by clopidogrel or its metabolites.

Aim 3: Evaluate the effects of clopidogrel in the posterior cerebral artery of $P2Y_{12}^{-/-}$ mice.

In the fourth chapter of this dissertation, I further evaluated clopidogrel's effect on the vasculature of $P2Y_{12}^{-/-}$ mice. I hypothesized that clopidogrel or its metabolites inhibit constriction upon stimulation of the $P2Y_2$ receptor in both wild-type and $P2Y_{12}^{-/-}$ mice. Additionally, I hypothesized that clopidogrel impaired the biomechanical properties of the

posterior cerebral artery. These studies elucidated whether clopidogrel's effects were $P2Y_{12}$-independent.

Conclusion

This introduction has described the current knowledge gaps surrounding the pleiotropic effects of clopidogrel. In summary, it is unclear how clopidogrel potentiates adverse bleeding independent from $P2Y_{12}$. The information within this introduction was used to formulate the central hypothesis that the clopidogrel prodrug or one of its metabolites modulates $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and/or $P2Y_{14}$ signaling in the vasculature to potentiate bleeding. The next chapter of this dissertation will elucidate which purinergic receptor is impaired by clopidogrel treatment.

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CHAPTER 2

Clopidogrel Treatment Inhibits $P2Y_2$-Mediated Constriction in the Rabbit Middle Cerebral Artery

Modified from: Kuszynski DS, Christian BD, Dorrance AM, Lauver DA. Clopidogrel treatment inhibits $P2Y_2$-Mediated constriction in the rabbit middle cerebral artery. Eur J Pharmacol. 2021 Nov 15;911:174545. doi: 10.1016/j.ejphar.2021.174545. Epub 2021 Oct 2. PMID: 34606835; PMCID: PMC8577565.

Abstract

Clopidogrel is an effective purinergic $2Y_{12}$ receptor ($P2Y_{12}$) antagonist used to prevent arterial thrombosis, but its use is associated with adverse bleeding. Clinical studies have demonstrated that clopidogrel users have an increased risk of cerebral microbleeds and intracerebral hemorrhage. My previous studies suggest that non-platelet mechanisms mediate these adverse bleeding events; I hypothesize that clopidogrel or one of its metabolites interacts with blood vessels directly to cause bleeding. New Zealand white rabbits (1.9-2.7 kg) were treated orally with vehicle or clopidogrel (3 or 10 mg/kg) for three days. On the fourth day, the rabbits were anesthetized for blood collection and then euthanized. The brain was collected, and the middle cerebral arteries were isolated. We used light transmission aggregometry and pressure myography to elucidate the mechanisms of the off-target effects associated with clopidogrel treatment. We confirmed that inhibition of $P2Y_{12}$ activation by clopidogrel inhibited ADP-induced platelet aggregation but had no impact on $P2Y_{12}$-independent arachidonic acid- or collagen-induced platelet aggregation. Analysis of middle cerebral arteries from clopidogrel-treated rabbits showed that clopidogrel did not affect $P2Y_4$, $P2Y_6$, and $P2Y_{14}$ receptor-mediated contraction but attenuated the contractile response after $P2Y_2$ receptor activation. Further analysis determined that $P2Y_2$-mediated constriction was endothelium-dependent. Vasoconstriction is a primary component of hemostasis, and impaired vasoconstriction can prolong bleeding. These results suggest clopidogrel inhibits the endothelial $P2Y_2$ receptor in the middle cerebral artery, which provides a mechanistic explanation for the adverse cerebral bleeding associated with the drug.

1. Introduction

Arterial thrombosis is the most common cause of death in the developed world [1]. Dual antiplatelet therapy is a frequently prescribed preventative treatment, consisting of low-dose aspirin in combination with a $P2Y_{12}$ antagonist. All $P2Y_{12}$ antagonists have bleeding risks; however, clopidogrel is preferred due to its comparative bleeding safety [2]. Although clopidogrel has the least risk, its use leads to a significant number of cerebral microbleeds and intracerebral hemorrhages [3–6]. This is a public health concern as more than 7000 patients per year in the United States suffer from potentially debilitating intracerebral hemorrhages resulting from antithrombotic therapies [6].

The ability of clopidogrel to promote bleeding is well described [2,4,7–14]. We showed that intravenous administration of clopidogrel produces bleeding without comparable platelet inhibition in rabbits [15]. André et al. observed increased bleeding in $P2Y_{12}^{-/-}$ mice treated with clopidogrel, compared to vehicle-treated $P2Y_{12}^{-/-}$ mice [14]. This suggests that the increase in bleeding is not $P2Y_{12}$-dependent.

Based on the evidence presented, it appears that the bleeding effects of clopidogrel cannot be explained entirely by the inhibition of platelets. Therefore, we must define clopidogrel's complete mechanism of action to understand the enhanced bleeding risk associated with its use. The metabolism of clopidogrel is complex [15,16]. Only about 5% of the prodrug is converted into the active metabolite responsible for inhibiting $P2Y_{12}$ [16], the remainder is converted into more than 15 metabolites which may have differential effects [14,17–20]. The target(s) with which the metabolites interact are not known; these unidentified targets could contribute to the adverse bleeding associated with clopidogrel.

Vascular dysfunction represents one possible explanation for clopidogrel-associated bleeding. Several purinergic receptors are expressed in vascular smooth muscle cells (VSMCs), including $P2Y_2$, $P2Y_4$, and $P2Y_6$. $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are expressed in cerebral artery endothelial cells [18]. Purinergic receptors are crucial in the maintenance of vascular function [18]. $P2Y_4$ and $P2Y_6$ regulate vascular tone, while $P2Y_2$ and $P2Y_4$ mediate

endothelial and VSMC proliferation and differentiation [21,22]. Under mechanical stress, uridine adenosine tetraphosphate (Up_4A) is released from endothelial cells to produce vasoconstriction via $P2Y_2$ receptor activation [23]. If clopidogrel metabolites antagonize $P2Y_2$ receptors, they could disrupt vasoconstriction.

The studies described above lead us to hypothesize that clopidogrel metabolites inhibit the vasoconstriction produced by purinergic receptor activation. Impaired vasoconstriction could lead to a local increase in flow thereby weakening the wall structure to ultimately cause bleeding. We investigated clopidogrel's effect on purinergic receptor activation in the middle cerebral artery (MCA) from New Zealand white rabbits. The MCA was selected because it is commonly associated with cerebrovascular bleeding events [24]. We found that clopidogrel treatment reduced the percentage of MCAs that generated myogenic tone but did not impair the degree of tone generation in the MCAs that did. Additionally, $P2Y_2$ receptor activation was significantly inhibited in MCAs from clopidogrel-treated rabbits. These results suggest clopidogrel, or its metabolites, inhibit $P2Y_2$ receptor vasoconstriction in the MCA, which provides a mechanistic explanation for the adverse bleeding associated with the drug.

2. Materials and Methods

2.1 Chemicals

S-clopidogrel sulfate was purchased from Cayman Chemical Co. (Ann Arbor, MI). Adenosine diphosphate (ADP), arachidonic acid (AA), and collagen were purchased from Chrono-log Corporation (Havertown, PA). Polyethylene glycol 400 and N, N-dimethylacetamide (DMA) were purchased from Millipore Sigma (St. Louis, MO). Ketamine and xylazine were purchased from Covetrus (Dublin, OH). Fatal Plus was purchased from Vortech Pharmaceuticals, LTD (Dearborn, MI). MRS 2365, MRS 2768, MRS 4062, MRS 2693, NF 546, MRS 2905, and 2MeSADP were purchased from TOCRIS Bio-Techne Corporation (Minneapolis, MN).

2.2 Animal Care and Use

The procedures used in this study were approved by the Institutional Animal Care and Use Committee (Animal Use Form 07/17-115). They were performed following Michigan State University guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Michigan State University Campus Animal Resources provided all veterinary care.

2.3 Surgical Preparation and Drug Administration

Seventy-eight male New Zealand white rabbits (1.9-2.7 kg) were obtained from Charles River Laboratories, Inc. (Wilmington, MA). Rabbits were acclimated for a minimum of 5 days and had free access to standard chow and fresh water. Rabbits were maintained on an automated 12/12-hour light/dark cycle with 7:00 am as the start of the light phase. Vehicle (n=30), 3 mg/kg clopidogrel (n=21) or 10 mg/kg clopidogrel (n=18) were administered via oral gavage for 3 days. These doses of clopidogrel inhibit platelet aggregation in rabbits [15], and they mimic the doses prescribed for human patients [25,26]. Clopidogrel was dissolved in DMA at 20 mg/ml and diluted in polyethyleneglycol-400 (PEG400). On the day of the study, 16 hours after the third and final dose, rabbits were anesthetized with ketamine (40 mg/kg, IM) and xylazine (5 mg/kg, IM). The surgical site was shaved, and the rabbits were placed on a 37°C heating pad. Isoflurane (1-3%, inhaled) was administered through a mask placed over the mouth and nose. The right jugular vein was isolated and instrumented with a polyethylene cannula for blood collection. Respiratory rate, lead II electrocardiogram, heart rate, and body temperature were monitored throughout the procedure. This procedure was performed on all rabbits in this study.

2.4 Blood Collection

Blood samples were collected from the jugular cannula into a syringe containing 3.2% sodium citrate as an anticoagulant (1:10 citrate to blood ratio) the morning after the last drug treatment for platelet aggregometry (see subsection 2.5).

2.5 Platelet Aggregation

Platelet reactivity was determined in platelet-rich plasma (PRP) obtained from whole blood samples using light transmission aggregometry. Whole blood samples were centrifuged at 150 g for 10 minutes at room temperature and the PRP was collected. The remaining non-platelet blood cells were then centrifuged at 1500 g at room temperature for 10 minutes to obtain the platelet-poor plasma (PPP) and the pellet was discarded. *Ex vivo* platelet aggregation was assessed using a 4-channel aggregometer (Chrono-log Corporation Model 700; Chrono-log Corporation, Havertown, PA). PRP was added to a cuvette and heated to 37°C for three minutes. PRP was then placed in the 4-channel aggregometer and was continually stirred (1200 RPM) and maintained at 37°C during the assay. Platelet agonists (ADP [20 µM], arachidonic acid [500 µM], or collagen [2 µg/mL]) were added individually to each cuvette, and the change in light transmission relative to PPP was recorded.

2.6 Pressure Myography

After blood collection, the rabbits were euthanized by intravenous administration of 1 ml Fatal Plus followed by thoracotomy. The brain was harvested and placed in cold Ca^{2+}-free physiological salt solution (PSS) containing 141.9 mM NaCl, 4.79 mM KCl, 1.12 mM KH_2PO_4, 1.79 mM $MgSO_4$•$7H_2O$, 109 mM HEPES, and 59 mM dextrose. The left and right MCAs were then dissected from the brain and cleaned of any connective tissue. Each MCA was cannulated on 320 µm steel cannulas in a custom-made cannulation chamber. A column-pully system was used to pressurize the arteries to an intraluminal pressure of 80 mmHg. A leak

test was performed before each experiment; only MCAs that held pressure were used for studies. The intraluminal pressure was then returned to 40 mmHg and Ca^{2+}-containing PSS containing 1.8 mM Ca^{2+}, 141.9 mM NaCl, 4.79 mM KCl, 1.12 mM KH_2PO_4, 1.79 mM $MgSO_4 \bullet 7H_2O$, 109 mM HEPES, and 59 mM dextrose was added to the circulating bath and warmed to 37°C. After 30 minutes, the MCA was pressurized to 90 mmHg and maintained a flow rate of 250 µl/min until the generation of myogenic tone. If the myogenic tone was not generated the artery was pre-constricted with 10^{-6} M serotonin. Once the lumen diameter stabilized, increasing concentrations of the selective purinergic agonists (2MeSADP, MRS 2365, MRS 2768, MRS 4062, MRS 2693, NF 546, and MRS 2905) were added to the luminal perfusate to assess vasoconstriction. These selective agonists were chosen to determine which specific receptor was impacted by clopidogrel treatment; endogenous ligands, such as ATP and UTP, would not elucidate the specific receptor inhibited by clopidogrel treatment. 2MeSADP displays selectivity for $P2Y_1$ (EC_{50} = 8.29 nM), $P2Y_{12}$ (EC_{50} = 9.05 nM) and $P2Y_{13}$ (EC_{50} = 19 nM). MRS2365 displays selectivity for $P2Y_1$ (EC_{50} = 0.4 nM). MRS 2768 displays selectivity for $P2Y_2$ (EC_{50} = 1.89 µM). MRS4062 displays selectivity for $P2Y_4$ (EC_{50} = 23 nM). MRS 2693 displays selectivity for $P2Y_6$ (EC_{50} = 0.015 µM). NF 546 displays selectivity for $P2Y_{11}$ (EC_{50} = 6.27 µM). MRS 2905 displays selectivity for $P2Y_{14}$ (EC_{50} = 0.92 nM). Only one agonist was evaluated in each MCA. The lumen diameter was measured using a 4X objective (Nikon Plan objective; Numerical Aperture: 0.25) with a Leica Leitz DM IL microscope. The average lumen diameter at each agonist concentration was recorded using VasoTracker software [27]. At the end of the experiment, ethylene glycol tetraacetic acid (EGTA; 2 mM) and sodium nitroprusside (SNP; 10 µM) were added to the bath to obtain a measure of the maximum dilation achievable by each MCA.

2.7 Removal of Endothelium

The MCA was isolated from vehicle-treated rabbits as described in subsection 2.6. To remove the endothelium, the lumen of each vessel was rubbed with a silk suture before cannulation.

After cannulation, an air bubble was passed through the lumen to ensure denudation of the endothelium. The MCA was then pressurized to 90 mmHg and maintained at a flow rate of 250 µl/min at 37°C with Ca^{2+}-containing PSS until the generation of myogenic tone. Once the MCA lumen diameter stabilized, 10^{-4} M carbachol was added to the luminal perfusate to test for complete denudation of the endothelium. After verifying the removal of the endothelium, increasing concentrations of ADP or MRS 2768 were added to the vessels (10^{-9}–10^{-5} M) to test the constriction response.

2.8 Statistical Analysis

Data were analyzed using GraphPad Prism version 9.0. Analysis of platelet aggregation was performed by one-way analysis of variance (ANOVA) and compared to the vehicle using Dunnett's post-test. The myogenic tone was analyzed for normality using the Shapiro-Wilk test and resulted in *p* values of 0.0370 and 0.0106 for 3 mg/kg and 10 mg/kg clopidogrel, respectively. The non-parametric Kruskal-Wallis one-way ANOVA was then performed and compared to the vehicle using Dunn's multiple comparison test. I then analyzed the proportion of MCAs that generated at least 15% myogenic tone using a Chi-square test. Constriction responses were analyzed by two-way ANOVA and compared to the vehicle using Dunnett's post-test. In all cases, results were considered significant at $p<0.05$. Significance was denoted as *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

3. Results

3.1 ADP-Induced Platelet Aggregation is Inhibited by Clopidogrel Treatment

As expected, ADP-induced platelet aggregation was inhibited by treatment with clopidogrel compared to vehicle (Figure 9A). AA acts via the cyclooxygenase-1 pathway and activates thromboxane A_2 receptors, while collagen activates the glycoprotein VI receptor; as expected,

neither signaling pathway was inhibited by clopidogrel treatment (Figures 9B and 9C, respectively). The collagen response was variable among animals which has been previously described in rabbits [15].

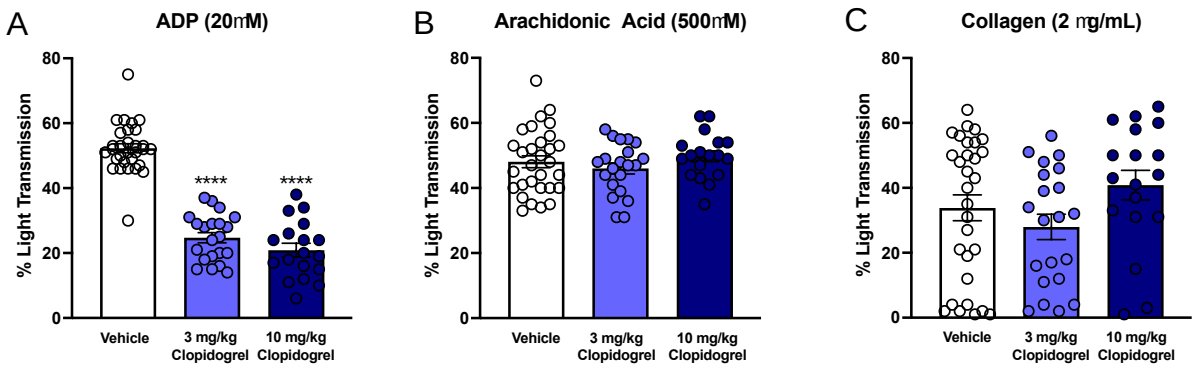


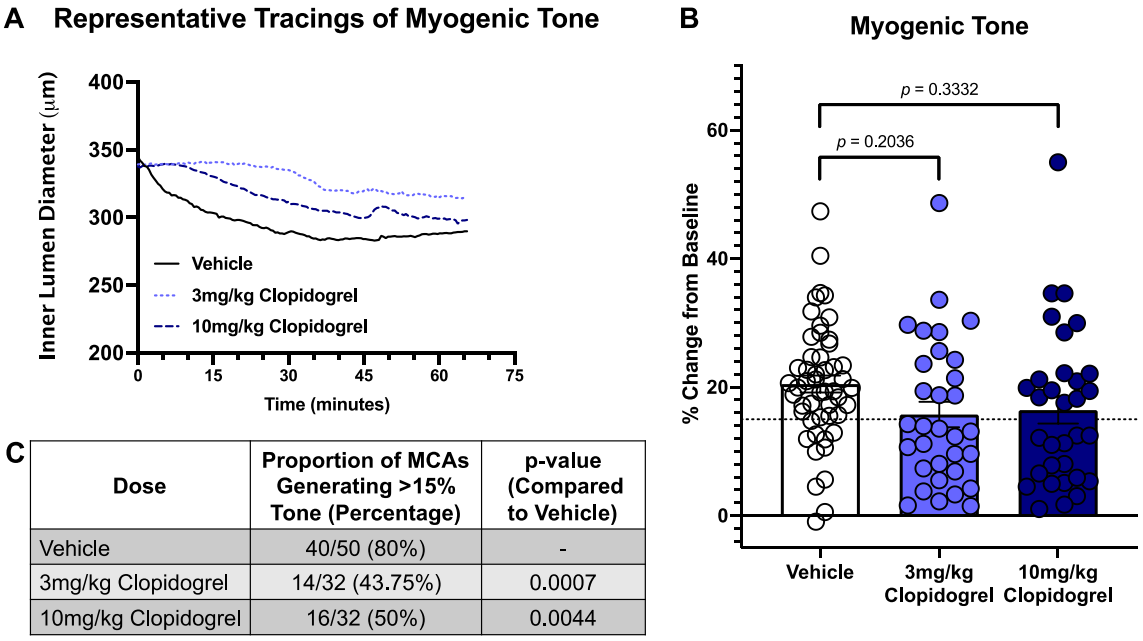
Figure 9: Platelet aggregation after clopidogrel treatment in rabbits. Platelet function was assessed by light transmission aggregometry using a 4-channel aggregometer. Percent ADP-induced (A), arachidonic acid (AA)-induced (B), and collagen-induced (C) platelet aggregation of blood collected from vehicle and clopidogrel (3 mg/kg and 10 mg/kg) treated rabbits. The data are presented as the mean ± SEM. ****p<0.0001 when compared with the vehicle-treated group by one-way ANOVA followed by Dunnett's post hoc test. Vehicle n=30, 3 mg/kg clopidogrel n=21, 10 mg/kg clopidogrel n=18.

3.2 Middle Cerebral Artery Purinergic Receptor Activation Response After Clopidogrel Treatment

3.2.1 Myogenic Tone Generation

There was no significant difference in the myogenic tone generation among treatment groups (Figures 10A and 10B). However, significantly more vehicle-treated MCAs generated at least 15% myogenic tone than clopidogrel-treated MCAs; 80% of vehicle-treated, 43.75% of 3mg/kg clopidogrel-treated, and 50% of 10mg/kg clopidogrel-treated MCAs generated more than 15% myogenic tone (Figure 10C). There was no significant difference between treatment groups of MCAs that generated at least 15% myogenic tone. The MCAs that generated at least 15% myogenic tone obtained a combined, over all treatment groups, constriction from baseline of 24.93% ± 0.9736% (vehicle: 23.86% ± 1.159%; 3 mg/kg clopidogrel: 27.03% ± 2.241%; 10

mg/kg clopidogrel: 25.82% ± 2.439%). The MCAs that did not generate at least 15% myogenic tone were preconstricted with 10^{-6} M serotonin to obtain a combined constriction from baseline of 30.42% ± 3.085% (vehicle: 28.88% ± 2.486%; 3 mg/kg clopidogrel: 29.80% ± 5.220%; 10 mg/kg clopidogrel: 31.65% ± 5.475%). There was no significant difference among treatment groups of MCAs that were preconstricted.



C

Dose	Proportion of MCAs Generating >15% Tone (Percentage)	p-value (Compared to Vehicle)
Vehicle	40/50 (80%)	-
3mg/kg Clopidogrel	14/32 (43.75%)	0.0007
10mg/kg Clopidogrel	16/32 (50%)	0.0044

Figure 10: Myogenic tone generation in the middle cerebral arteries from rabbits. Percent myogenic tone generation of the middle cerebral artery after pretreatment with vehicle and clopidogrel (3 and 10 mg/kg). The artery was pressurized to 90 mmHg, Ca^{2+}-PSS flow rate was maintained at 250 µl/min and 37ºC. A) Representative tracings of myogenic tone generation taken from VasoTracker software. B) Maximal stable (15 min) myogenic tone generation. The data are presented as the mean ± SEM. The data were compared with the vehicle-treated group by Kruskal-Wallis one-way ANOVA followed by Dunn's multiple comparison test. The *p*-values are reported above each comparison. The dashed line indicates a 15% myogenic tone. C) Proportion of MCAs that generated at least 15% myogenic tone. The data were compared to the vehicle-treated group by the Chi-square test. Vehicle n=50, 3 mg/kg clopidogrel n=32, 10 mg/kg clopidogrel n=32.

3.2.2 P2Y Agonist Mediated Vascular Reactivity

MCA vascular reactivity was assessed using the following P2Y-selective agonists: MRS 2365 – a $P2Y_1$ agonist, MRS 2768 – a $P2Y_2$ agonist, MRS 4062 – a $P2Y_4$ agonist, MRS 2693 – a

$P2Y_6$ agonist, NF 546 – a $P2Y_{11}$ agonist, MRS 2905 – a $P2Y_{14}$ agonist, and 2MeSADP – a $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ agonist. These agonists were added to the luminal perfusate. Activation of $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{14}$ induced dose-dependent constriction of the MCA (Figure 11). $P2Y_1$ and $P2Y_{11}$ selective agonists did not induce any response in the MCA (Figure 12). These findings suggest that $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{14}$ are present and functional in the MCA, while $P2Y_1$ and $P2Y_{11}$ are not.

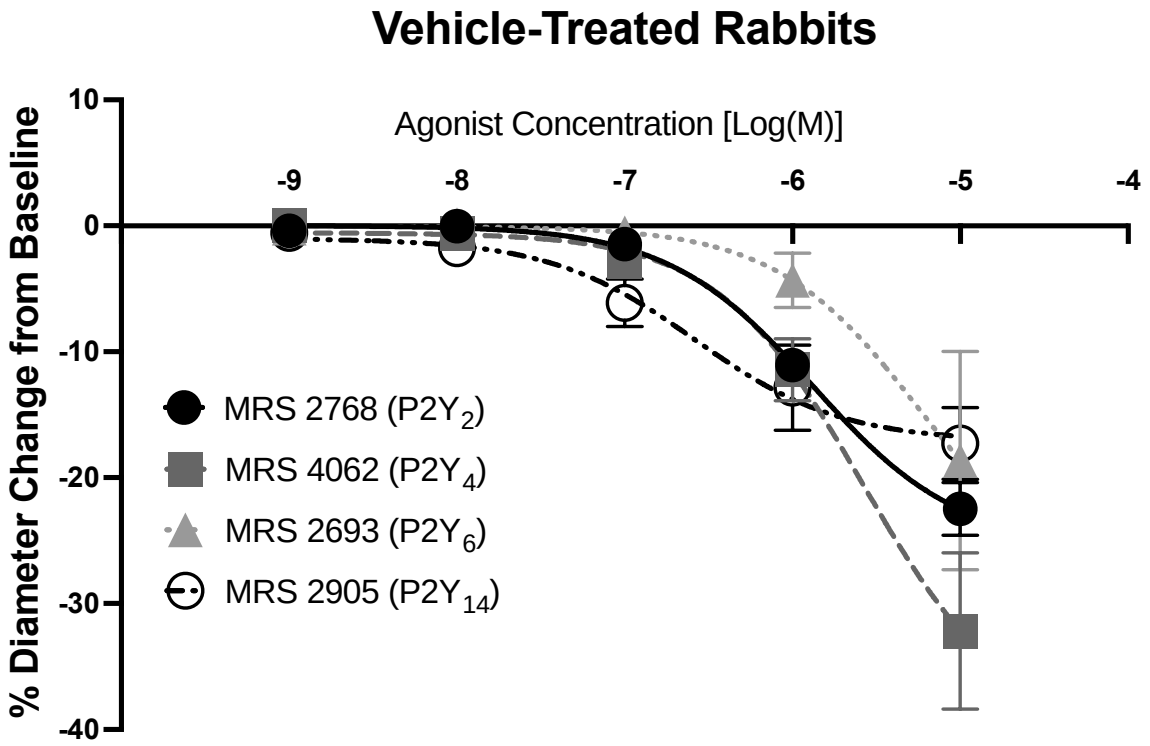


Figure 11: Purinergic receptor activation from vehicle-treated rabbits. P2Y mediated concentration-dependent constrictor responses in the MCA from vehicle-treated rabbits. Constriction response was evaluated by pressure myography. Circles, solid line: MRS 2768 a $P2Y_2$-selective agonist; squares, dashed line: MRS 4062 a $P2Y_4$-selective agonist; triangles, dotted line: MRS 2693 a $P2Y_6$-selective agonist; open circles, dashed and dotted line, MRS 2905 a $P2Y_{14}$-selective agonist. The data are presented as the mean ± SEM. The data were compared with the vehicle-treated group by two-way ANOVA followed by Dunnett's post hoc test. n=5 per group.

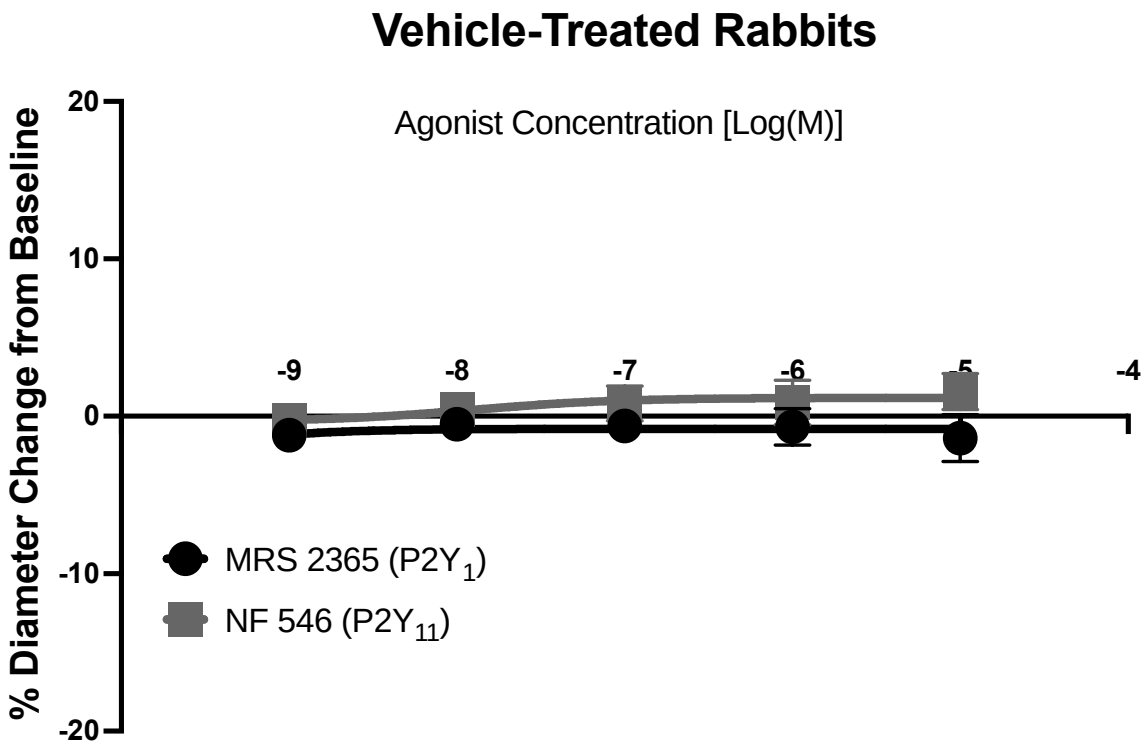


Figure 12: $P2Y_1$ and $P2Y_{11}$ activation in the middle cerebral arteries. There were no $P2Y_1$ or $P2Y_{11}$ mediated responses in the MCA from vehicle-treated rabbits. The MCA responses were evaluated by pressure myography. Black circles, MRS 2365, a $P2Y_1$-selective agonist; grey squares, NF546, a $P2Y_{11}$-selective agonist. The data are presented as the mean ± SEM and compared with the vehicle-treated group by two-way ANOVA followed by Dunnett's post hoc test. n=5 per group.

Clopidogrel treatment (3 and 10 mg/kg) did not affect the vascular responses induced by activation of $P2Y_4$ (Figure 13A), $P2Y_6$ (Figure 13B), or $P2Y_{14}$ (Figure 13C). The contractile response to the $P2Y_2$ agonist was attenuated in MCAs from clopidogrel (3 and 10 mg/kg) treated rabbits (Figure 14). This finding suggests clopidogrel or its metabolites interact with the $P2Y_2$ receptor signaling pathway in the MCA.

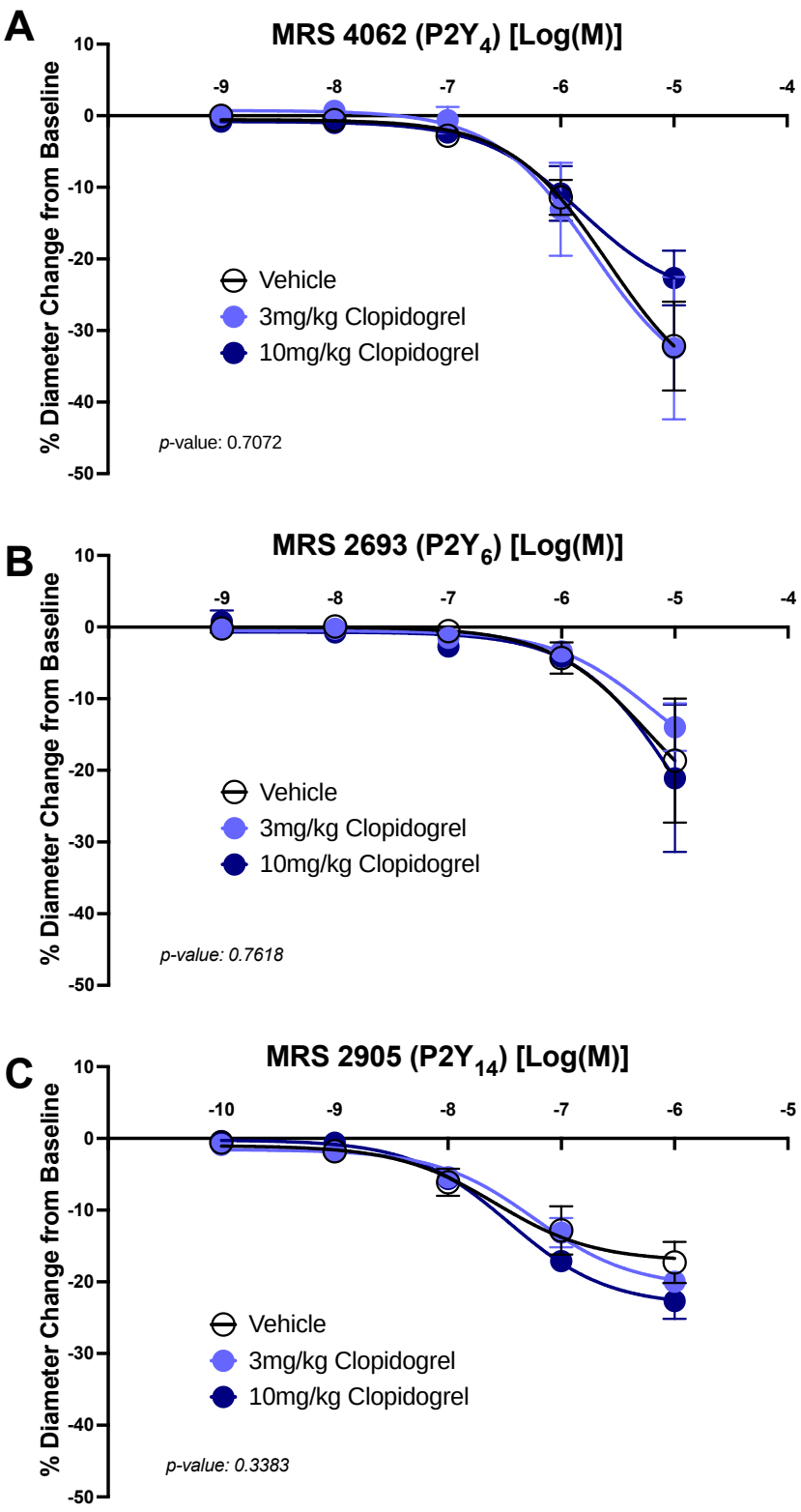


Figure 13: $P2Y_4$, $P2Y_6$, and $P2Y_{14}$ activation in the middle cerebral arteries. The effects of clopidogrel on $P2Y_4$, $P2Y_6$ and $P2Y_{14}$ mediated contraction in MCAs. The constriction responses were evaluated by pressure myography. Activation by A) MRS 4062, a $P2Y_4$-selective agonist, B) MRS 2693, a $P2Y_6$-selective agonist, and C) MRS 2905, a $P2Y_{14}$-selective agonist after vehicle and clopidogrel (3 and 10 mg/kg. The data are presented as the mean ± SEM and compared with the vehicle-treated group by two-way ANOVA followed by Dunnett's post hoc test. n=5 per group.

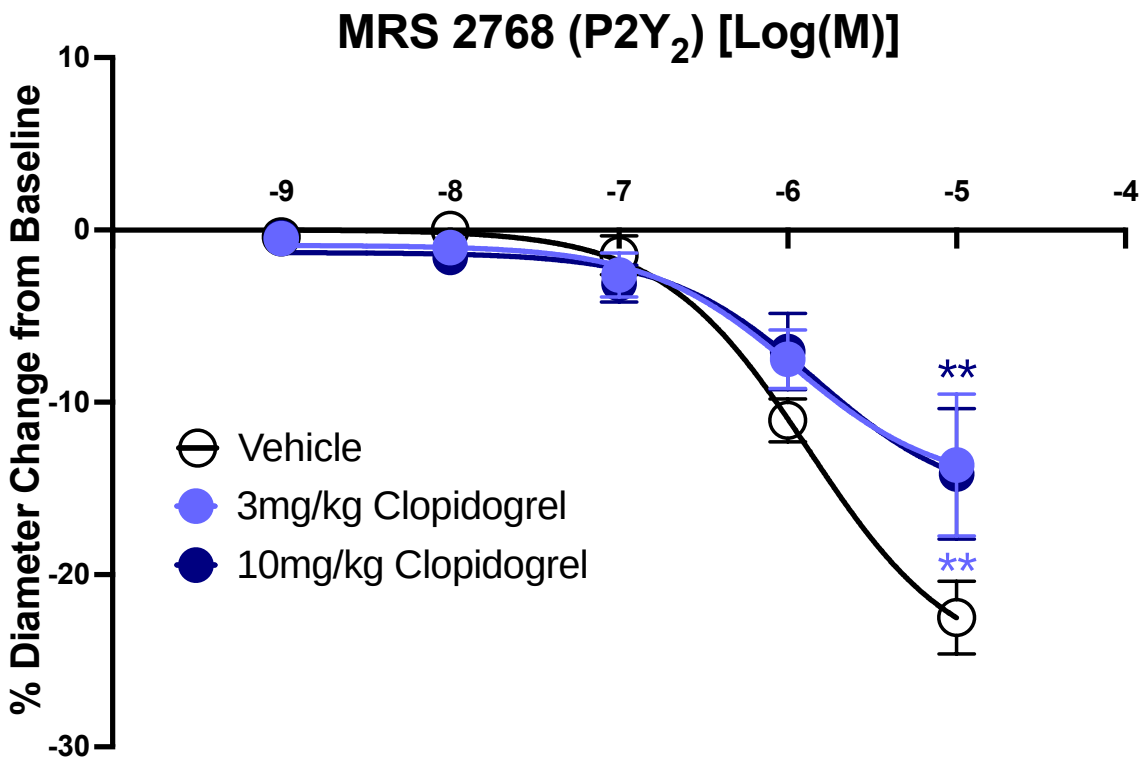


Figure 14: $P2Y_2$ activation in the middle cerebral arteries. The effects of clopidogrel on $P2Y_2$ mediated contraction in MCAs. Constriction response was evaluated by pressure myography. Activation by MRS 2768, a $P2Y_2$-selective agonist after vehicle and clopidogrel (3 and 10 mg/kg) treatment. X-axis is Log[agonist] M. The data are presented as the mean ± SEM. ***p*<0.01 when compared with the vehicle-treated group by two-way ANOVA followed by Dunnett's post hoc test. n=5.

Next, we determined the effects of clopidogrel on the vasculature in response to 2MeSADP. 2MeSADP is unable to be hydrolyzed to form adenosine like ADP. This allows for selective receptor activation of $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors. Treatment with antiplatelet doses of clopidogrel (3 and 10 mg/kg) did not affect the activation by 2MeSADP (Figure 15). These findings suggest clopidogrel treatment does not impair MCA response through $P2Y_1$, $P2Y_{12}$, or $P2Y_{13}$ receptor signaling pathways.

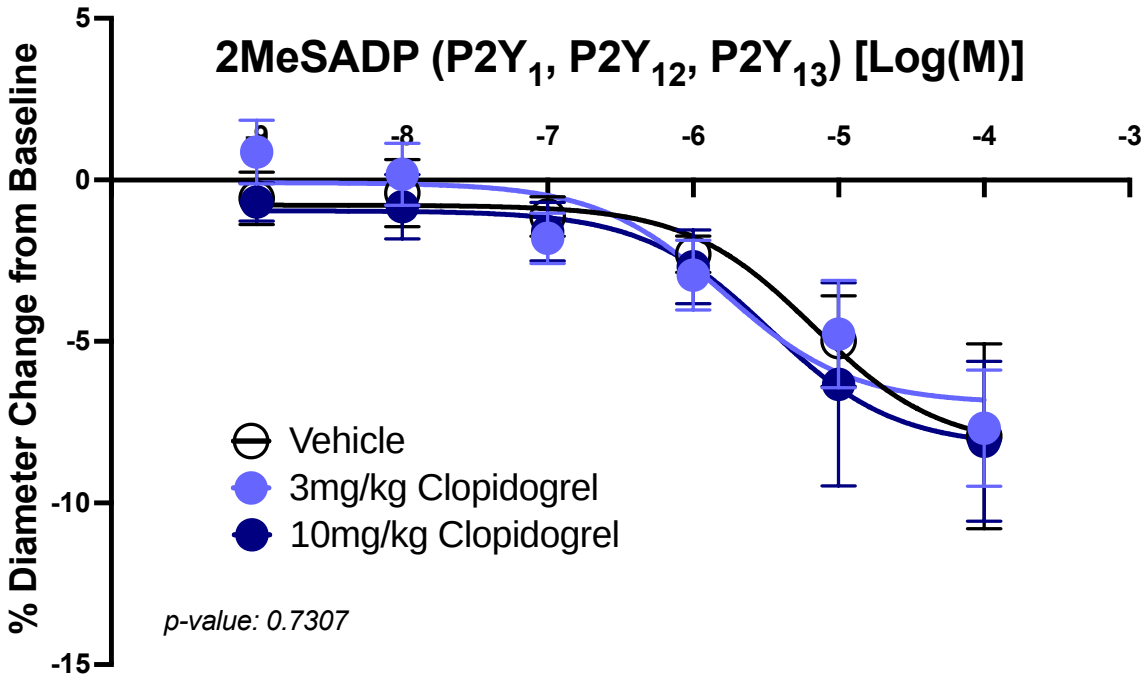


Figure 15: $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ activation in the middle cerebral arteries. The effects of clopidogrel on 2MeSADP mediated contraction in MCAs. The constriction responses were evaluated by pressure myography. Activation by 2MeSADP, a $P2Y_1$-, $P2Y_{12}$-, and $P2Y_{13}$-selective agonist after vehicle and clopidogrel (3 and 10 mg/kg) treatment. The data are presented as the mean ± SEM and compared with the vehicle-treated group by two-way ANOVA followed by Dunnett's post hoc test. n=5 per group.

3.2.3 $P2Y_2$ Activation is Endothelium Dependent

In the MCA, luminal ADP-activation induced vasodilation. To evaluate the location of the dilation response we removed the endothelium. Dilation of the MCA by ADP (10^{-9}–10^{-5}M) was assessed. There was a slight decrease in ADP-induced dilation after removing the endothelium; however, this was not statistically significant (Figure 16A). This finding suggests ADP-induced vasodilation could be both endothelium- and VSMC-dependent.

In the MCA, $P2Y_2$ activation induced vasoconstriction. Since our purinergic agonists were added through the lumen, we predicted that the contraction was mediated by endothelial purinergic receptors. To evaluate the localization of the receptors activated by MRS 2768 we removed the endothelium. There was no change in myogenic tone between endothelium removed and endothelium intact groups (data not shown). Constriction of the MCA by MRS

2768 (10^{-9}–10^{-5} M) was assessed. Removal of endothelium significantly attenuated the vasoconstriction response (Figure 16B). This finding suggests activation of $P2Y_2$-mediated vasoconstriction is endothelium-dependent.

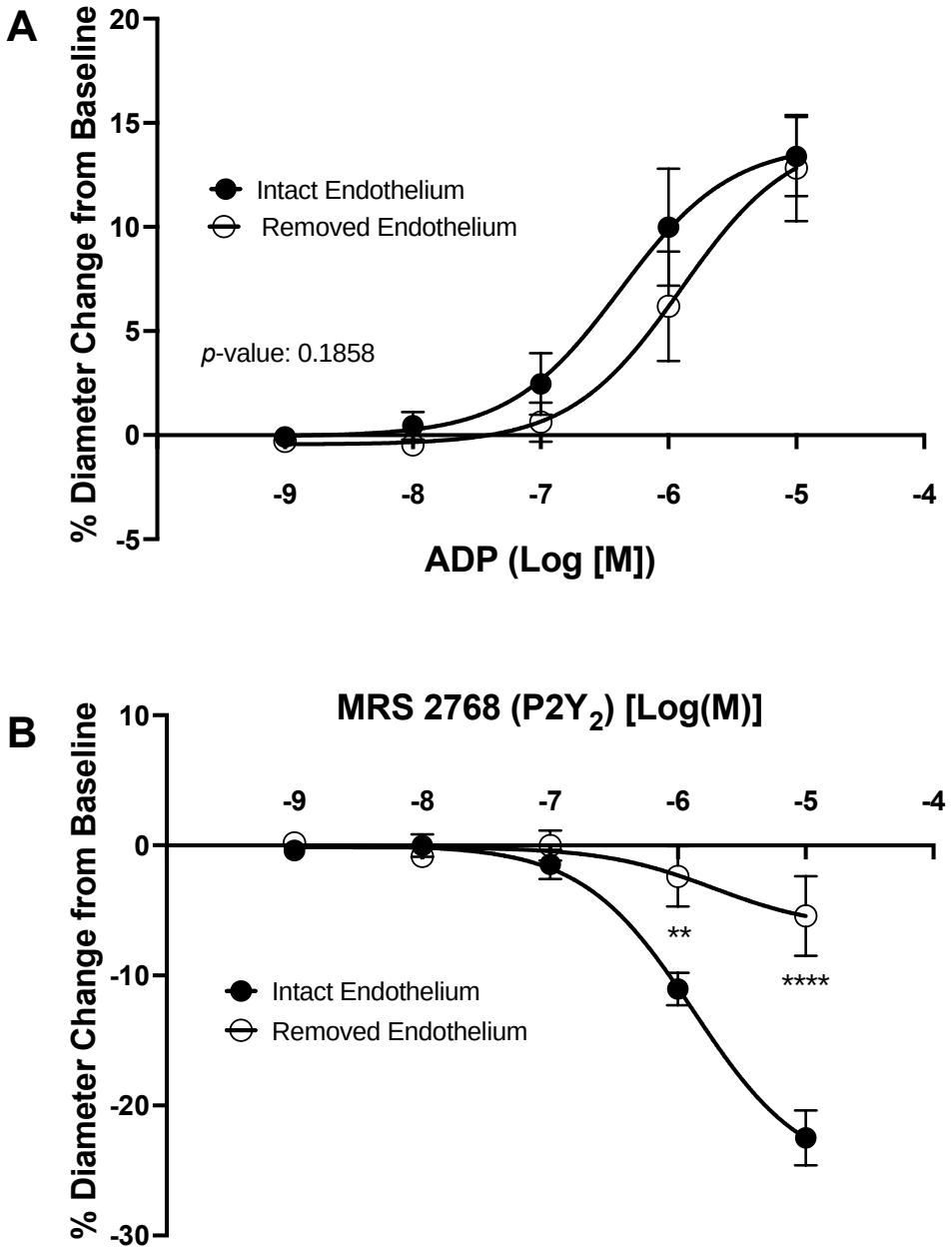


Figure 16: $P2Y_2$ activation in deendothelialized middle cerebral arteries. (A) ADP-induced dilation and (B) $P2Y_2$ mediated contraction in intact or deendothelialized MCAs. The constriction responses were evaluated by pressure myography. Activation by ADP, a non-selective agonist, or MRS 2768, a $P2Y_2$-selective agonist after vehicle treatment. The data are presented as the mean ± SEM. **p<0.01 and ****p<0.0001 when compared with the vehicle-treated group by two-way ANOVA followed by Dunnett's post hoc test. n=5 per group.

4. Discussion

The novel findings in this study are that 1) clopidogrel pretreatment did not significantly inhibit myogenic tone generation in MCAs but did reduce the percentage of MCAs that generated tone, 2) clopidogrel significantly inhibited vasoconstriction resulting from $P2Y_2$ receptor activation, and 3) $P2Y_2$-mediated vasoconstriction is endothelium-dependent in the rabbit MCA. Taken together, our data suggest clopidogrel, or its metabolites, inhibit the endothelial $P2Y_2$ receptor in the cerebral vasculature. We propose that this is the mechanism by which adverse cerebral bleeding events occur with clopidogrel treatment.

Platelet aggregation studies confirmed clopidogrel inhibits ADP-induced aggregation without modifying AA- and collagen-induced platelet aggregation (Figure 9A, 9B, and 9C, respectively). The results verify that clopidogrel selectively inhibits the $P2Y_{12}$ receptor on platelets and has no detectable off-target effects in platelets. These experiments also prove that the doses of clopidogrel used in the current study were clinically relevant.

Clopidogrel is associated with an increased risk of intracerebral hemorrhage [10,13]. Off-target effects of clopidogrel are frequent enough for them to be a significant concern [14,17,19,20]. Several lines of evidence suggest clopidogrel metabolites have secondary biological effects. As described above, clopidogrel-treated $P2Y_{12}^{-/-}$ mice have increased blood volume loss compared to vehicle-treated $P2Y_{12}^{-/-}$ mice [14]. Clopidogrel directly induces vasorelaxation in rat caudal arteries without hepatic metabolism [19] and causes nitric oxide-dependent vasodilation in isolated guinea-pig hearts [20]. Lastly, one of the few characterized secondary metabolites of clopidogrel, M15, prolongs the time to occlusion after $FeCl_3$ carotid artery injury-induced thrombosis in mice, indicating at least one additional metabolite of clopidogrel is antithrombotic [28]. Importantly, M15 does not inhibit $P2Y_{12}$ platelet aggregation (unpublished data from our laboratory). Despite this knowledge, the mechanism by which clopidogrel metabolites disrupt normal hemostatic function is unknown.

Cerebral autoregulation is the process through which cerebral arteries maintain cerebral blood flow (CBF) in response to changing perfusion pressure [29]. The endothelium contributes to

autoregulation through its mechanoreceptor properties, and shear stress induces endothelium-dependent vasoconstriction [29]. When mean arterial pressure is increased, cerebral arteries contract to increase their resistance to flow and maintain CBF at a constant level. Dysregulated CBF or excessive CBF can cause injury resulting in blood-brain barrier breakdown, leading to hemorrhage. The MCA is responsible for regulating downstream blood flow in the parenchymal arterioles, which penetrate the brain tissue. If CBF is not regulated in the MCA, it could cause damage to the parenchymal arterioles, resulting in bleeding.

Vascular resistance and myogenic tone generation control CBF, and impairments in these processes cause structural abnormalities, such as weakening of the vessel wall [29,30]. Myogenic tone is a state of partial constriction which is activated by the pressure-induced stretch of the VSMCs [31]. Myogenic tone dysregulation causes brain injuries due to elevated hydrostatic pressure in the microcirculation [32–34]. Parenchymal arterioles treated with antisense oligonucleotides for $P2Y_4$ and $P2Y_6$ had a decreased myogenic tone response; however, treatment with antisense oligonucleotides for $P2Y_2$ did not impair myogenic tone [21]. This indicates that $P2Y_4$ and $P2Y_6$ are critical regulators of myogenic tone in parenchymal arterioles in mice. In this study, we report our findings comparing the myogenic tone response of MCAs from rabbits treated with vehicle and clopidogrel (3 and 10 mg/kg; Figure 10B). A significant percentage of the MCAs from clopidogrel-treated rabbits did not generate the expected amount of tone in the MCA (15%; Figure 10C). When the MCAs that did not generate more than 15% tone were removed from the analysis, there was no significant difference in the myogenic tone generation between the groups. The same was true if the MCAs with low tone remained in the analysis. These findings suggest clopidogrel, or its metabolites, may interact with one of the receptors important in generating myogenic tone; however, it remains unclear why this effect was not observed in all the clopidogrel-treated rabbits. Although $P2Y_4$ and $P2Y_6$ are important in myogenic tone generation, clopidogrel pretreatment did not affect the constriction response upon activation of either of these receptors (Figure 13). These findings suggest that the mechanism by which clopidogrel

impairs myogenic tone generation is likely unrelated to $P2Y_4$ or $P2Y_6$. We are the first to report myogenic tone generation in the MCAs from rabbits. I found that myogenic tone generation in rabbit MCAs was variable. All procedures were performed uniformly to decrease variability.

The myogenic tone data presented here are clinically significant assuming that a loss of tone drives the incidence of cerebral bleeding events in clopidogrel patients. Interestingly, the percentage of the rabbits that lost the ability to generate tone in the MCA after clopidogrel treatment closely mimics the portion of human patients that experience clopidogrel-associated cerebral bleeding (cerebral microbleeds occur in 30-50% of patients taking clopidogrel for at least one year) [4,9]. I recognize that further studies are required to definitively link the loss of myogenic tone with increased cerebral bleeding in patients.

The key observation from my results was that clopidogrel treatment inhibited the vasoconstriction response produced by $P2Y_2$ receptor activation. This suggests clopidogrel, or its metabolites, inhibit the $P2Y_2$ receptor to impair vasoconstriction, which is important in regulating CBF. During mechanical stress, $P2Y_2$ activation is required for vasoconstriction [23]. Vasoconstriction reduces blood flow to reduce blood loss during vascular injury, and impaired vasoconstriction can lead to excessive bleeding. $P2Y_2$ has also been reported to regulate endothelial and smooth muscle cell proliferation [22]. Therefore, impaired $P2Y_2$ activation by clopidogrel treatment would modulate both acute vascular processes (vasoconstriction) and long-term vascular remodeling (cell proliferation) [22,23]. However, it is unlikely that I have observed any long-term effects in this study due to the treatment time.

The selective purinergic agonists used in these experiments triggered constriction of the MCA. The effects of purinergic activation in the endothelium vary substantially among vascular beds. Purinergic receptor activation is commonly known to cause dilation by endothelium-derived nitric oxide generation in large cerebral arteries [35,36]. However, I observed a constriction response upon the addition of the purinergic agonists. I hypothesized that perhaps the agonists diffused through the endothelium to activate VSMC purinergic receptors to evoke this constriction response. I tested this hypothesis by removing the endothelium from another

group of MCAs. Upon removal of the endothelium, there was a significant decrease in vasoconstriction of the MCA in response to MRS 2768. While I am not the first to report $P2Y_2$ vasoconstriction [37], the mechanism to which endothelial $P2Y_2$ activation causes constriction is still unknown. Recent reports indicate uridine adenosine tetraphosphate (Up_4A) activates $P2Y_2$ to induce vasoconstriction [38,39]. Additionally, when endothelial cells are subjected to sudden stretch, activation of purinergic receptors causes the release of Up4A and other endothelium-derived contracting factors (EDCFs). EDCFs diffuse to the VSMCs and initiate contraction [40]. The mechanism to which activation of endothelial $P2Y_2$, via MRS 2768 or Up4A, results in constriction needs to be assessed in future studies.

One limitation of this study is that the duration of clopidogrel treatment does not accurately reflect the clinical situation. However, we demonstrated steady-state platelet inhibition after 3 days of treatment, suggesting a maximal clopidogrel effect. By observing acute changes in vascular function associated with this dosing regimen, we have elucidated potential mechanisms underlying long-term bleeding side effects observed with clopidogrel use. Additionally, we did not investigate changes in vascular permeability. Loss of vascular integrity is indicative of a higher risk of vascular leakage and intracerebral hemorrhage [30]. Future studies will determine whether there is a decrease in vascular integrity after clopidogrel treatment. Finally, while I analyzed the consequence of purinergic receptor activation with selective agonists, the specific location where these receptors are expressed (endothelium or VSMC) is unknown in the MCA of rabbits. The specific site(s) where these receptors are expressed needs to be examined.

In conclusion, in an experimental vessel model in rabbits, clopidogrel pretreatment did not impair myogenic tone generation but did impair the percentage of MCAs that generated at least 15% myogenic tone. Furthermore, clopidogrel significantly inhibited vasoconstriction resulting from activation of the $P2Y_2$ receptor. Our results suggest that clopidogrel inhibits the endothelial $P2Y_2$ receptor in the cerebral vasculature which could explain the adverse bleeding side effects observed clinically.

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CHAPTER 3

Evaluation of Clopidogrel Metabolites to Determine the Cause of Adverse Bleeding

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Abstract

Clopidogrel is an effective inhibitor of $P2Y_{12}$ platelet activation, however, it is associated with cerebral microbleeds and intracerebral hemorrhages. I previously discovered that clopidogrel treatment inhibited $P2Y_2$-mediated vasoconstriction, which might partially explain the adverse bleeding associated with clopidogrel use. The objective of the current study was to evaluate clopidogrel and its key metabolites (the M1 metabolite, the M2 metabolite, and the M13 metabolite) to elucidate which metabolite(s) were responsible for inhibiting $P2Y_2$. P2Y receptor subtypes were stably transfected into 1321N1 astrocytoma cells which were used to determine specific inhibition of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$ receptors. Calcium mobilization was assessed by Fluo-4 Direct™ Calcium Dye after treatment with clopidogrel, M1, M2, a conjugate of M13, and ticagrelor (another $P2Y_{12}$ antagonist which does not require metabolic bioactivation). Of the drugs used, only ticagrelor and the M13 conjugate inhibited $P2Y_{12}$-induced calcium mobilization. Clopidogrel, M1, M2, M13 conjugate, and ticagrelor did not inhibit $P2Y_1$-, $P2Y_2$-, $P2Y_4$-, or $P2Y_6$-induced calcium mobilization. To test the effects of clopidogrel and its metabolites on bleeding, seventy-two male C57Bl/6 mice were randomized to one of 4 treatment groups: vehicle, 10 mg/kg clopidogrel, 10 mg/kg M1, or 10 mg/kg M2. Tail amputation blood loss and platelet activation were assessed after 5-day oral administration. Treatment with clopidogrel and M2 inhibited platelet activation while only treatment with clopidogrel increased tail blood loss. These results suggest the prodrug clopidogrel, the M1 metabolite, and the M2 metabolite are not responsible for the inhibition of $P2Y_2$ or adverse bleeding.

1. Introduction

Clopidogrel, an antiplatelet agent, is frequently prescribed to prevent arterial thrombosis. The active metabolite of clopidogrel covalently binds the $P2Y_{12}$ receptor on platelets which inhibits their activation, leading to a decrease in clot formation. All $P2Y_{12}$ antagonists have bleeding risks due to their effect on hemostasis. However, clopidogrel has the least risk and is, therefore, the preferred agent for long-term treatment [1,2]. Despite the relative safety of clopidogrel, its bleeding risk remains significant. Clopidogrel use is associated with serious cerebral bleeding consequences like cerebral microbleeds and intracerebral hemorrhage [2–9]. The exact mechanism by which these spontaneous bleeding events occur is unknown, but they cannot be completely explained by the inhibition of $P2Y_{12}$ because clopidogrel treatment increases bleeding even in $P2Y_{12}$ knockout animals [10–12]. To better understand clopidogrel-associated bleeding, we must more clearly define clopidogrel's actions in the body.

Vascular dysfunction represents one possible explanation for clopidogrel-associated bleeding. Several purinergic receptors are expressed in vascular smooth muscle cells (VSMCs) and endothelial cells. The $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are proposed to be expressed on both the VSMCs and endothelium. Additionally, the $P2Y_1$ receptor is suggested to be expressed only on the endothelium [13–15]. These receptors have been reported to be crucial in the maintenance of vascular function and the induction of vasoconstriction [11,13,15,16]. Therefore, modulation of these receptors by clopidogrel, or its metabolites, could pose a threat to normal vascular function. Vasoconstriction represents an important component of the hemostatic process [17,18]. Upon vascular injury, several chemical mediators are released to induce vasoconstriction, thereby reducing blood flow and blood loss. An impairment in vasoconstriction could lead to an increase in bleeding. Our laboratory previously investigated clopidogrel's effect on purine-dependent vascular function in the middle cerebral artery (MCA) by treating rabbits for 3 days with clopidogrel or vehicle [11]. Clopidogrel treatment of rabbits significantly inhibited endothelium-dependent $P2Y_2$-mediated vasoconstriction in MCAs *ex*

vivo. This mechanism may partially explain the adverse cerebral bleeding events that occur with clopidogrel treatment.

Clopidogrel metabolism is complex resulting in the generation of at least 17 metabolites [19–23]. The active clopidogrel metabolite, M13, covalently modifies cysteine residues on $P2Y_{12}$ to inhibit platelet activation [24]. The pharmacology of the additional clopidogrel metabolites has not been extensively investigated, although there is ample evidence suggesting meaningful biological effects [11,12,25–27]. Interestingly, a conjugate of M13 did not significantly increase bleeding [10]. This suggests that the other metabolites of clopidogrel, and not the M13 metabolite, are responsible for the increase in bleeding.

There are eight subtypes of P2Y receptors that are activated by adenine or uracil nucleotides. The $P2Y_2$ and $P2Y_{11}$ receptors are nonselective between adenine and uracil nucleotides. However, the $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors are selective for adenine nucleotides, and the $P2Y_4$, $P2Y_6$, and $P2Y_{14}$ receptors are selective for uracil nucleotides. Interestingly, the nucleotide that activates each receptor does not correlate with the signal transduction for that receptor. The $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ receptors are G_q-coupled, the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors are G_i-coupled, and the $P2Y_{11}$ receptor is G_s-coupled. Activation of each of these receptors directly relates to calcium release from a cell. Activation of the G_q-coupled purinergic receptors increases calcium through activation of phospholipase C. The activation of G_i-coupled purinergic receptors increases calcium through the inhibition of adenylyl cyclase. Alternatively, activation of the G_s-coupled P2Y receptor activates adenylyl cyclase to decrease calcium release. While activation of each of these receptors causes a different response, the P2Y receptor family members are structurally similar to one another [28–30]. Additionally, the binding pockets on P2Y receptors are similar, therefore providing evidence for the binding of the same endogenous nucleotide [28]. Furthermore, competition binding experiments demonstrate the ability of agonists and antagonists to bind multiple P2Y receptor family members [31].

The studies described above lead me to hypothesize that a metabolite of clopidogrel, other than M13, is responsible for inhibiting $P2Y_2$ to cause adverse bleeding. Long-term impairment of $P2Y_2$ vasoconstriction can prompt a local increase in flow thereby weakening the wall structure to ultimately cause bleeding [17,18,32–34]. The inhibition of $P2Y_2$ activation, and subsequent calcium release, by an additional metabolite, would explain the adverse bleeding observed with clopidogrel treatment, but not seen with treatment of the M13 conjugate. We investigated the effect of the clopidogrel prodrug, the M1 metabolite, the M2 metabolite, an M13 conjugate, and ticagrelor (an alternative $P2Y_{12}$ antagonist) on calcium mobilization in $P2Y_{12}$, $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ stably transfected 1321N1 astrocytoma cells. The 1321N1 astrocytoma cell line does not endogenously express P2Y receptors, making them ideal for transfection of single P2Y receptors for further analysis [35]. We confirmed ticagrelor and the M13 conjugate inhibited $P2Y_{12}$ calcium mobilization. Unsurprisingly, clopidogrel did not inhibit $P2Y_{12}$ calcium mobilization. Clopidogrel requires extensive metabolism, not available in cell culture, to generate M13. Unexpectedly, clopidogrel, M1, M2, the M13 conjugate, and ticagrelor did not affect $P2Y_1$, $P2Y_2$, $P2Y_4$, or $P2Y_6$ calcium mobilization. To further analyze metabolite response, clopidogrel, M1, M2, and vehicle treatment were given to mice to measure blood loss and platelet inhibition. Clopidogrel and M2 significantly decreased platelet activation while only clopidogrel increased blood loss, yet there was no change with M1 treatment. Together, these results suggest the prodrug clopidogrel, M1, and M2 are not responsible for the adverse bleeding side effect associated with clopidogrel.

2. Materials and Methods

2.1 Chemicals

S-clopidogrel sulfate was purchased from TCIchemicals (Portland, OR). SR26334 (M1) was purchased from Synthonix (Wake Forest, NC). Ticagrelor was purchased from Cayman Chemical (Ann Arbor, MI). M2 was synthesized and purified by the Medicinal Chemistry Core at Michigan State University (East Lansing, MI). The M13 conjugate was synthesized, purified,

and donated by Dr. Haoming Zhang (Ann Arbor, MI). Adenosine diphosphate (ADP) was purchased from Chrono-log Corporation (Havertown, PA). MRS 2693 and 2MeSADP were purchased from TOCRIS Bio-Techne Corporation (Minneapolis, MN). UTP, G418, 1321N1 astrocytoma cells, glutathione, Drabkin's reagent, and hemoglobin human were purchased from Millipore Sigma (Burlington, MA). Dulbecco's Modified Eagle's Medium and Lipofectamine 2000 was purchased from ThermoFisher Scientific (Waltham, MA). 1321N1 stably transfected astrocytoma cells ($P2Y_1$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$) were purchased from Kerafast (Boston, MA). 2X Fluo-4 Direct™ Calcium Dye was purchased from Molecular Probes (Eugene, OR).

2.2 Cell Culture

The pLXSH-HA-$P2Y_2$ plasmid was donated by Dr. Robert Nicholas (University of North Carolina). $P2Y_2$ cDNA (3 µg) was transfected into 1321N1 cells using Lipofectamine 2000 with 1% G418 selection. $P2Y_2$ stably transfected cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum and 0.5% G418. $P2Y_1$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$ stably transfected cells (Kerafast) were cultured in DMEM with 10% fetal bovine serum. The cells were then incubated in 5% CO_2 at 37°C until 90% confluent. Cells were examined regularly and monitored for growth and contamination.

2.3 Ca^{2+} Mobilization Assay

Cells were plated in black, clear-bottom 384-well plates at a concentration of 10,000 cells/well. The cells were allowed to attach overnight and the following day 2X Fluo-4 Direct™ Calcium Dye containing 1 µM glutathione (GSH) was added directly to each well containing cells in culture media. The plate was incubated at 37°C for 30 minutes. Then clopidogrel, M1, M2, and ticagrelor were added at increasing concentrations to predetermined wells. The plate was incubated for another 30 minutes before measuring fluorescence in response to agonists,

MRS 2693 ($P2Y_6$-selective agonist), UTP ($P2Y_2$- and $P2Y_4$-selective agonist), and 2MeSADP ($P2Y_1$-, $P2Y_{12}$-, and $P2Y_{13}$-selective agonist). Fluorescence was measured on the FDSS/μCell Functional Drug Screening System (Hamamatsu) for excitation at 494nm and emission at 516nm. The max amplitude fluorescent ratio was analyzed.

2.4 Animal Care and Use

The procedures used in this study were approved by the Institutional Animal Care and Use Committee (protocol number 202000209). They were performed under Michigan State University guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Michigan State University Campus Animal Resources provided all veterinary care.

2.5 Drug Administration and Surgical Preparation

Drugs were suspended in Nutella. Vehicle, 10 mg/kg clopidogrel, 10 mg/kg M1, or 10 mg/kg M2 was given to mice orally every day for 5 days. On the 6^{th} day, mice were anesthetized with 3% isoflurane. Mice were then used for blood loss measurements or blood collection (see subsections 2.6 and 2.7).

2.6 Blood Loss Measurements

Once mice were anesthetized, the tail was amputated 5 mm from the tip using a number 10 surgical blade. The mouse was then immediately placed in a horizontal position, and the tail was immersed in 1.5 ml of Drabkin's reagent for 30 minutes. Drabkin's reagent lyses erythrocytes and releases hemoglobin which reacts irreversibly with potassium cyanide and potassium ferricyanide to form the stable pigment cyanmethemoglobin [36]. A standard hemoglobin curve was generated using human hemoglobin with known concentrations.

Hemoglobin concentrations from tail blood loss were measured on a Tecan Infinite M1000 Pro Microplate Reader at 540 nm. Data were analyzed using iControl software and then computed to the human hemoglobin curve.

2.7 Blood Collection

Once mice were anesthetized, blood was collected via cardiac puncture into a syringe containing heparin as an anticoagulant (1:10 150 Units/ml heparin to blood ratio) for further analysis of platelet activation (see subsection 2.8).

2.8 Platelet Activation

Platelets were identified using anti-CD42d binding, and platelet activation was determined by Jon/A in whole blood stimulated by ADP. Heparinized blood (50 µL) was washed with 200 µL Tris-Hep buffer and with 1 mL Tyrode's buffer. Blood was centrifuged at 900 rpm for 5 minutes. The pellet was resuspended in 1.25 mL Tyrode's buffer, and $CaCl_2$ (1 mM) was added. The washed blood (25 µL) was then incubated with ADP (20 µM), or Tyrode's buffer for 2 minutes. Jon/A (PE) and anti-CD42d (APC) were then added to these samples and incubated for 15 minutes in the dark. Flow cytometric assessment was performed using a BD Accuri C6 (BD Biosciences) available in the MSU South Campus Flow Cytometry Core Facility. Events (20,000) were collected on a log scale for FSC-A, gated on the CD42d positive platelet scatter-based population and size, followed by doublet discrimination. Positive platelets were quantified as a measure of platelet activation. Data were analyzed using CFlow Plus software, v1.0.227.04 (BD Biosciences).

2.9 Statistical Analysis

Data were analyzed using GraphPad Prism version 9.0. Calcium mobilization was analyzed by two-way analysis of variance (ANOVA) and compared to the vehicle using Dunnett's post-test. Analysis of blood loss and platelet activation were performed by one-way ANOVA and compared to the vehicle using Dunnett's post-test. In all cases, results were considered significant at $p<0.05$. Significance was denoted as $*p<0.05$, $**p<0.01$, $***p<0.001$, and $****p<0.0001$.

3. Results

3.1 Calcium Mobilization

3.1.1 $P2Y_{12}$ Calcium Mobilization is Inhibited by Ticagrelor and a M13 conjugate

Agonist-induced calcium mobilization was assessed in $P2Y_{12}$ stably transfected 1321N1 cells after clopidogrel, M1, M2, the M13 conjugate, or ticagrelor treatment. Ticagrelor (1 µM and 10 µM) and the M13 conjugate (10 µM) inhibited calcium release in $P2Y_{12}$ stably transfected 1321N1 cells (Figure 17). Clopidogrel, M1, and M2 had no effect. Since clopidogrel requires metabolic activation by cytochrome P450s (CYP450s) to generate M13, and this cell line does not have endogenous CYP450 activity, M13 is not generated. This finding suggests ticagrelor and M13 inhibits $P2Y_{12}$, while the prodrug clopidogrel and the clopidogrel metabolites: M1 and M2, do not inhibit $P2Y_{12}$.

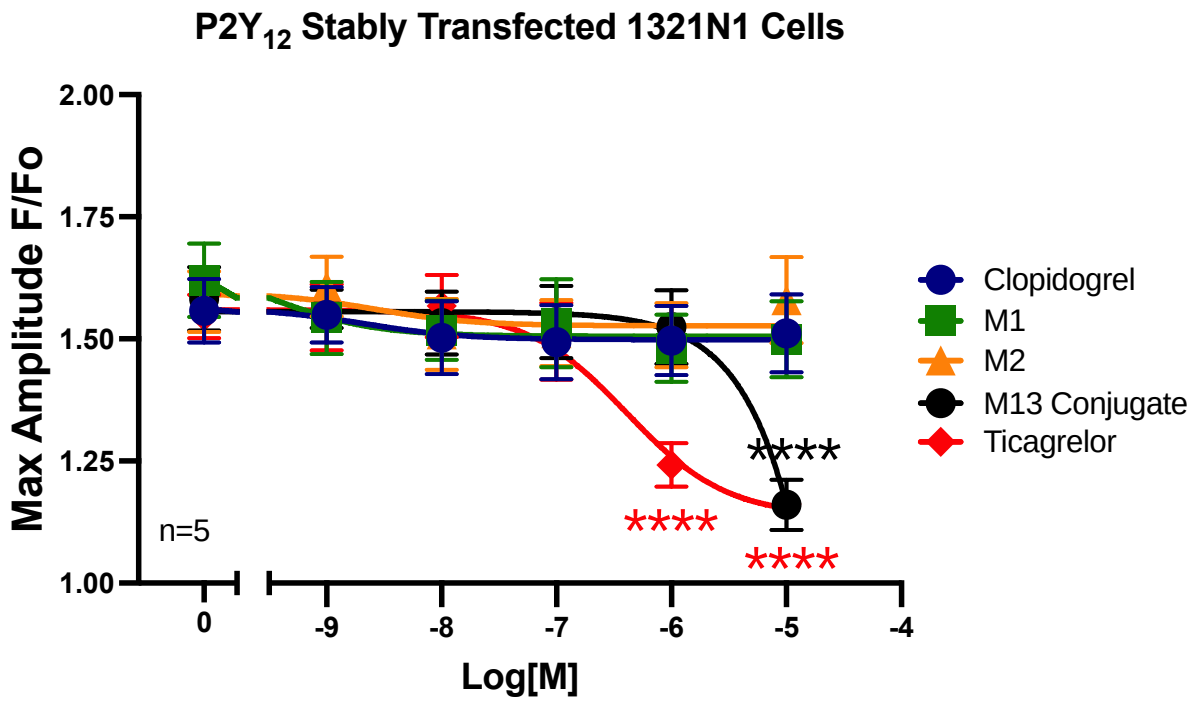

Figure 17: Calcium mobilization in P2Y$_{12}$ 1321N1 astrocytoma cells. Concentration-response curves of clopidogrel, M1, M2, the M13 conjugate, and ticagrelor using the calcium mobilization assay on P2Y$_{12}$-stably transfected 1321N1 cells. 2MeSADP (10µM) was used for receptor activation. The data represent the mean of five experiments ± SEM. ****p<0.0001 when compared with vehicle-treatment by two-way ANOVA followed by Dunnett's post hoc test.

3.1.2 P2Y$_1$, P2Y$_2$, P2Y$_4$, and P2Y6 Calcium Mobilization is not Inhibited by Clopidogrel or Clopidogrel Metabolites

Agonist-induced calcium mobilization was next assessed in P2Y$_1$, P2Y$_2$, P2Y$_4$, and P2Y$_6$ stably transfected 1321N1 cells after clopidogrel, M1, M2, or ticagrelor treatment (Figure 18). P2Y$_1$ stably transfected cells were stimulated by 2MeSADP. Clopidogrel, M1, M2, and ticagrelor did not inhibit P2Y$_1$ (Figure 18A). P2Y$_2$ and P2Y$_4$ stably transfected cells were stimulated by UTP. Clopidogrel, M1, M2, the M13 conjugate, and ticagrelor had no effect in either cell type (Figure 18B and 18C). Lastly, P2Y$_6$ stably transfected cells were stimulated by the selective agonist, MRS 2693. Clopidogrel, M1, M2, and ticagrelor did not inhibit P2Y$_6$ (Figure 18D). These findings suggest the prodrug clopidogrel, ticagrelor, and the clopidogrel metabolites, M1 and M2, do not inhibit P2Y$_1$, P2Y$_2$, P2Y$_4$, and P2Y$_6$.

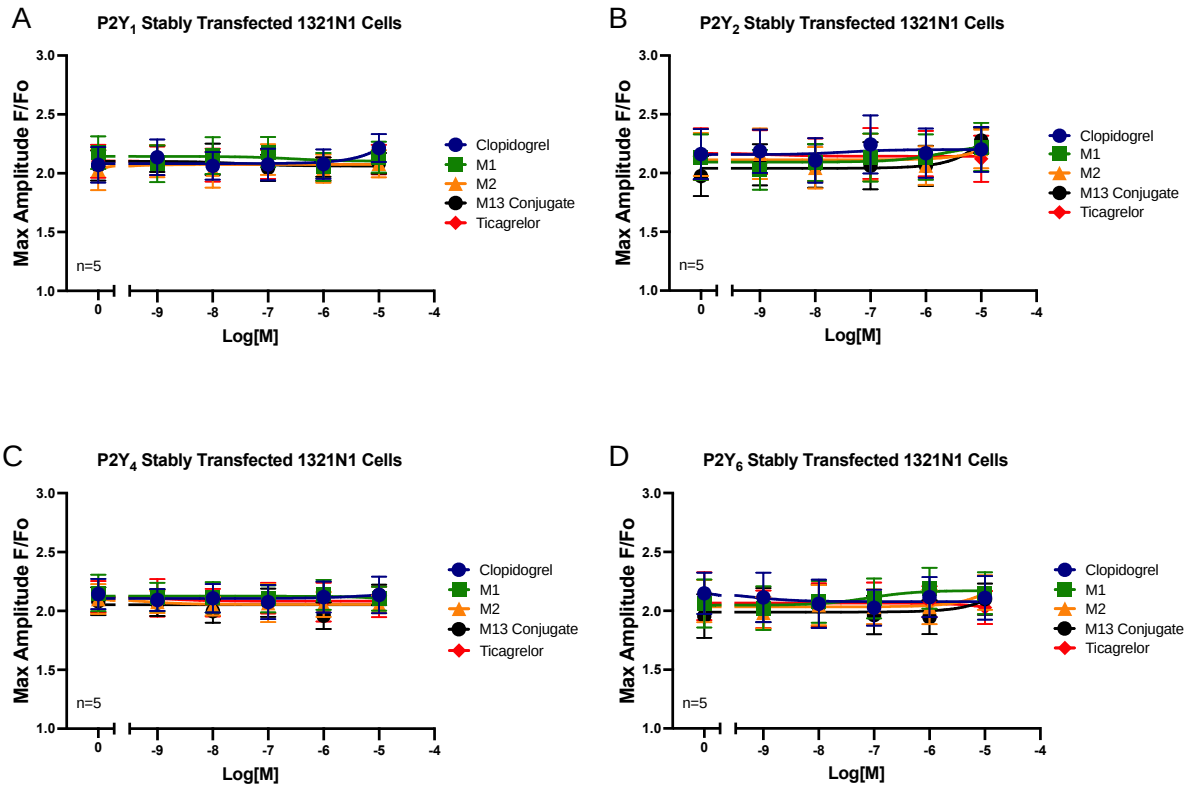

Figure 18: Calcium mobilization in $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ 1321N1 astrocytoma cells. Concentration-response curves of clopidogrel, M1, M2, the M13 conjugate, and ticagrelor using the calcium mobilization assay on A) $P2Y_1$-, B) $P2Y_2$-, C) $P2Y_4$-, and D) $P2Y_6$-stably transfected 1321N1 cells. 2MeSADP (10 µM) was used for receptor activation of $P2Y_1$. UTP (10 µM) was used for receptor activation of $P2Y_2$ and $P2Y_4$. MRS 2693 (10 µM) was used for receptor activation of $P2Y_6$. The data represent the mean of five experiments ± SEM. Compared with the vehicle-treatment by two-way ANOVA followed by Dunnett's post hoc test.

3.2 Clopidogrel Treatment Increases Blood Loss in Mice

We next studied the effects of 10 mg/kg clopidogrel, 10 mg/kg M1, and 10 mg/kg M2 on blood loss and compared their effects to vehicle treatment. Hemoglobin was measured and the concentration was calculated against a standard curve generated using known human hemoglobin concentrations. Clopidogrel treatment significantly increased hemoglobin concentration (Figure 19). Treatment with 10 mg/kg of M1 or M2 metabolites did not

significantly increase hemoglobin concentration. These results suggest that M1 and M2 are not the metabolites responsible for the increase in bleeding seen with clopidogrel treatment.

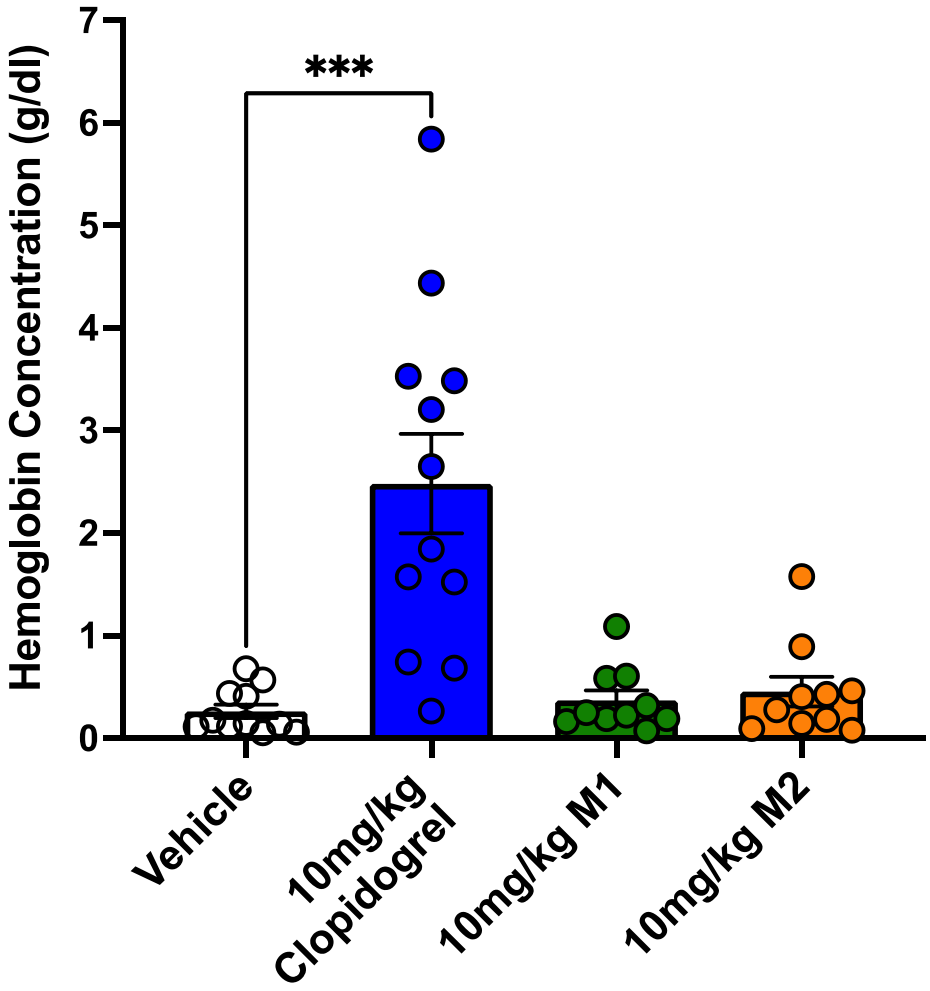

Figure 19: Blood loss after clopidogrel, M1, and M2 treatment. Blood loss hemoglobin concentrations in mice after 5-day treatment with vehicle, 10 mg/kg clopidogrel, 10 mg/kg M1, and 10 mg/kg M2. Blood was collected in Drabkin's reagent for 30 minutes and hemoglobin concentration was measured. The data represent the mean of twelve mice ± SEM. ****p*<0.001 when compared with the vehicle-treated group by one-way ANOVA followed by Dunnett's post hoc test.

3.3 Clopidogrel Treatment Inhibits Platelet Activation

The effects of vehicle, 10 mg/kg clopidogrel, 10 mg/kg M1, and 10 mg/kg M2 treatment on the activation of integrin $\alpha_{IIb}\beta_3$ in mice were measured by flow cytometry. Integrin $\alpha_{IIb}\beta_3$ expression was detected by Jon/A (PE). Treatment with 10 mg/kg clopidogrel or 10 mg/kg M2 decreased

the formation of integrin $\alpha_{IIb}\beta_3$ on platelets after ADP activation compared to vehicle (Figure 20). The M1 metabolite did not change the formation of integrin $\alpha_{IIb}\beta_3$. These results suggest that while clopidogrel and M2 inhibit platelet activation through the generation of M13, the M1 metabolite did not alter platelet activation and therefore did not generate M13.

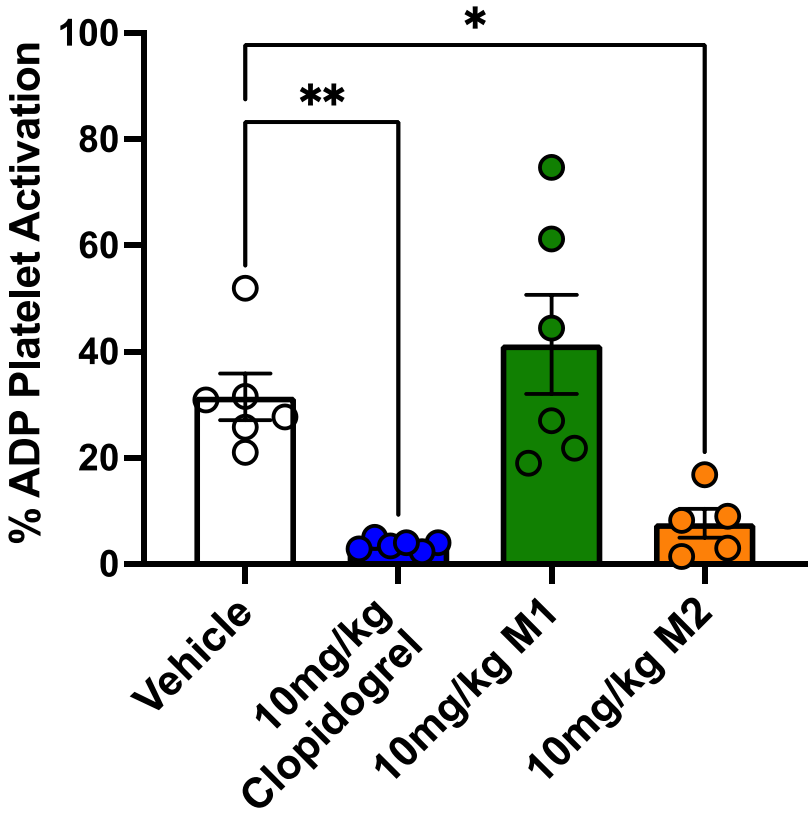

Figure 20: Platelet activation after clopidogrel, M1, and M2 treatment. Flow cytometric assessment of platelets activated with ADP after 5-day treatment with vehicle, 10 mg/kg clopidogrel, 10 mg/kg M1, and 10 mg/kg M2. The data represent the mean of six mice ± SEM. **p*<0.05 ***p*<0.01 when compared with the vehicle-treated group by one-way ANOVA followed by Dunnett's post hoc test.

4. Discussion

The novel findings in this study are that 1) the prodrug clopidogrel, the M1 metabolite, and the M2 metabolite do not inhibit $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{12}$, and 2) the M1 metabolite and the M2 metabolite do not cause an increase in blood loss. Taken together, our results suggest the prodrug clopidogrel, the M1 metabolite, and the M2 metabolite are not responsible for the inhibition of $P2Y_2$-mediated vasoconstriction and the adverse bleeding associated with clopidogrel treatment. I propose one of the other metabolites of clopidogrel is responsible for the adverse bleeding and inhibition of $P2Y_2$.

Clopidogrel treatment is an effective $P2Y_{12}$ antagonist prescribed to prevent thrombotic events. Although multiple $P2Y_{12}$ antagonists are available, clopidogrel has the least risk of bleeding [1,2]. Despite the relative safety of clopidogrel, serious bleeding events still occur such as cerebral microbleeds and intracerebral hemorrhage [3–6]. Our group previously reported an increase in bleeding with clopidogrel treatment but not with DT-678, a conjugate of M13 [10]. This indicated the adverse bleeding associated with clopidogrel treatment was not in response to the M13 metabolite and that the additional metabolites of clopidogrel are responsible for these undesired effects. In addition, our group demonstrated clopidogrel's ability to inhibit $P2Y_2$-mediated vasoconstriction [11]. We proposed that the prodrug clopidogrel, or its metabolites, increase cerebral bleeding events by inhibiting $P2Y_2$-mediated vasoconstriction. The goal of the current study was to determine which metabolite of clopidogrel is responsible for $P2Y_2$ inhibition and bleeding.

P2Y stably transfected 1321N1 cells confirmed that ticagrelor inhibits $P2Y_{12}$ and that the prodrug clopidogrel, M1, and M2 did not inhibit $P2Y_{12}$ (Figure 17). We also determined that the prodrug clopidogrel, M1, and M2 did not inhibit $P2Y_1$, $P2Y_2$, $P2Y_4$, or $P2Y_6$ (Figure 18). Thus, these results suggest the inhibition of $P2Y_2$ observed in the previous study was not due to the clopidogrel prodrug, the M1 metabolite, or the M2 metabolite.

In the current study, blood loss was significantly increased in mice treated with clopidogrel, but not in mice treated with the metabolites M1 or M2 (Figure 19). M2 can be further metabolized into at least 7 metabolites, including M13. These results suggest the adverse bleeding associated with clopidogrel is not due to M1, M2, or any of the 7 metabolites generated from M2. However, the production of each metabolite was not evaluated. To confirm M2 is further metabolized into M13, we assessed platelet activation after vehicle, clopidogrel, M1, or M2 treatment (Figure 20). Clopidogrel- and M2-treatment significantly inhibited platelet activation, however, M1 did not inhibit platelet activation. These results verify clopidogrel and M2 are metabolized into M13. Furthermore, this indicates that M2 and M13 are not responsible for the adverse bleeding associated with clopidogrel treatment. Based on this finding, the metabolites independent from M2 metabolism, M3-M10, should be assessed for adverse bleeding.

In conclusion, in a $P2Y_{12}$ stably transfected cell line, the clopidogrel prodrug, M1 and M2 did not inhibit calcium mobilization. Additionally, the clopidogrel prodrug, M1, and M2 did not inhibit calcium mobilization in $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ stably transfected cell lines. In an experimental model in mice, clopidogrel pretreatment increased blood loss, while M1 and M2 did not. Furthermore, clopidogrel- and M2-treatment inhibited platelet activation, while M1 did not affect platelet activation. Our results suggest M1 and M2 are not responsible for $P2Y_2$ inhibition. The additional metabolites of clopidogrel must be assessed individually to determine which metabolite is responsible for $P2Y_2$ inhibition and the increase in bleeding.

Acknowledgments

I would like to thank Edmund Ellsworth and Matthew Giletto for the development of M2. The generation of M2 was funded through the Molecular Discovery Grant. I would also like to thank Matt Bernard for his help in the development of the Flow Cytometry assay.

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CHAPTER 4

Clopidogrel Treatment and Loss of $P2Y_{12}$ Impair the Biomechanical Properties of the Posterior Cerebral Arteries in Mice

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Abstract

The favored $P2Y_{12}$ antagonist, clopidogrel, is associated with adverse bleeding, specifically cerebral microbleeds and intracerebral hemorrhage. Previous studies have demonstrated both non-platelet and non-$P2Y_{12}$ mechanisms that potentiate the adverse bleeding. Our group has observed $P2Y_2$ inhibition by clopidogrel treatment in New Zealand white rabbits. I hypothesize the adverse effects of clopidogrel also occur in animals deficient in $P2Y_{12}$, and that $P2Y_2$ inhibition by clopidogrel potentiates bleeding in $P2Y_{12}$ knockout ($P2Y_{12}^{-/-}$) mice. Wild-type, heterozygous $P2Y_{12}$ knockout, and homozygous $P2Y_{12}$ knockout C57/Bl6N mice (16-18 weeks) were treated orally with vehicle or 10 mg/kg clopidogrel for five days. On the sixth day, the mice were anesthetized for blood collection, blood loss measurements, and/or cerebral blood flow imaging. The mice were then euthanized, and the brain was collected. The posterior cerebral arteries were isolated for pressure myography. We also used platelet flow cytometry, laser speckle imaging of pial arteries, and hemoglobin concentration measurements to elucidate the mechanisms of off-target effects associated with clopidogrel treatment. I verified that clopidogrel treatment and the knockout of $P2Y_{12}$ inhibit ADP-induced platelet aggregation. Furthermore, clopidogrel and the knockout of $P2Y_{12}$ induce blood loss. Analysis of the biomechanical properties in posterior cerebral arteries demonstrated that $P2Y_{12}^{-/-}$ mice and clopidogrel-treated mice have decreased distensibility and increased arterial stiffness. Surprisingly, I did not observe $P2Y_2$ inhibition in the posterior cerebral arteries from clopidogrel-treated wild-type or $P2Y_{12}^{-/-}$ mice. These findings suggest posterior cerebral arteries could be more susceptible to clopidogrel-induced intracerebral hemorrhage through changes to the biomechanical properties of the vasculature. Interestingly, clopidogrel treatment in $P2Y_{12}^{-/-}$ mice may have protective effects as I did not observe significant differences in distensibility or arterial stiffness compared to vehicle-treated wild-type mice.

1. Introduction

The most common cause of death in the developed world is arterial thrombosis, which can cause serious problems such as heart attack, transient ischemic attack, and stroke [1]. Every 40 seconds someone in the United States suffers a stroke [1]. While most cardiovascular diseases are declining, stroke prevalence is expected to increase by 3.4 million individuals from 2012-2030 due to the aging population [2].

Dual antiplatelet therapy is the most common preventative treatment for arterial thrombosis. It consists of low-dose aspirin in combination with a $P2Y_{12}$ antagonist. The most commonly prescribed $P2Y_{12}$ antagonist, clopidogrel, is often chosen because of its safety and efficacy over other $P2Y_{12}$ antagonists. However, clopidogrel is still associated with adverse bleeding, particularly cerebral microbleeds and intracerebral hemorrhages [3–5]. Cerebral microbleeds are characterized as round or oval hypointense lesions <10 mm in diameter on MRI and are indicative of hemorrhage-prone small vessel pathology, which could ultimately contribute to intracerebral hemorrhage [6]. Cerebral microbleeds typically occur in patients with cerebrovascular diseases, such as stroke and hypertension [7]. Additionally, cerebral microbleeds are more frequent in antiplatelet users compared to non-antiplatelet users [4,6,8]. Of patients who have been on clopidogrel for at least one year, 30-40% had cerebral microbleeds, and patients who were on an antiplatelet agent for more than 5 years showed an increased risk of not only cerebral microbleeds but also macroscopic bleeding [5,9]. Most commonly, cerebral microbleeds are sealed by activation of hemostatic factors, thereby avoiding clinical symptoms [10]. Unfortunately, when patients are on antithrombotic therapy, these normal hemostatic mechanisms are suppressed, thus resulting in prolonged bleeding and an increased prevalence of intracerebral hemorrhage [9,11]. This is a significant public health concern as more than 7000 patients a year in the United States suffer potentially debilitating intracerebral hemorrhages, resulting from antithrombotic therapies [11].

I have previously reported that clopidogrel significantly increases bleeding time. Clopidogrel at low doses (3 mg/kg) increases bleeding time before platelet inhibition is detected [12]. This

suggests that the increase in adverse bleeding is not associated with the platelet $P2Y_{12}$ antagonistic effect of the drug. Factors other than platelets must be evaluated to determine the cause of clopidogrel bleeding. Of the P2Y receptor family members, $P2Y_2$, $P2Y_4$, and $P2Y_6$ are proposed to be expressed in vascular smooth muscle cells (VSMCs) while $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ are suggested to be expressed in the endothelial cells of cerebral arteries [13,14]. Samples are often contaminated with platelets so it is difficult to determine tissue-specific expression. Clopidogrel or one of clopidogrel's several metabolites could modulate one of these other purinergic receptors, thereby impairing artery function.

Upon vessel injury, there are three mechanisms for hemostasis. First, the vessel will immediately constrict in response to the release of endothelin from endothelial cells to reduce blood flow. This is quickly followed by platelet activation and platelet adhesion through the exposure of collagen. Additionally, the coagulation cascade is activated through the release of tissue factors from fibroblasts, leukocytes, and damaged vessels. This mechanism forms a stable clot to stop bleeding. I previously reported clopidogrel's ability to inhibit $P2Y_2$-mediated vasoconstriction [15]. The inhibition of $P2Y_2$-mediated vasoconstriction could be responsible for the adverse bleeding associated with clopidogrel use by inhibiting the first step in hemostasis, vasoconstriction.

The studies described above lead me to hypothesize that clopidogrel-associated bleeding results from non-$P2Y_{12}$-mediated mechanisms. The inhibition of $P2Y_2$ in the cerebral vasculature could explain the adverse bleeding that is observed clinically. To further elucidate this mechanism, this study investigated clopidogrel's effect on blood loss, platelet activation, cerebral blood flow, the biomechanical properties of the vessel, and vasoconstriction in the posterior cerebral artery (PCA) in $P2Y_{12}$ knockout ($P2Y_{12}^{-/-}$) mice. This will determine if these adverse effects are still observed in the absence of $P2Y_{12}$. I found that while clopidogrel treatment and loss of $P2Y_{12}$ in mice increased blood loss, decreased platelet activation, and induced structural changes to the vasculature, it did not affect cerebral blood flow or vasoconstriction. These results suggest that in mice, the loss of $P2Y_{12}$ and clopidogrel

treatment do not affect $P2Y_2$ vasoconstriction, but do impair the biomechanical properties of the posterior cerebral arteries, which provides an additional mechanistic explanation for the adverse bleeding associated with the drug.

2. Materials and Methods

2.1 Chemicals

S-clopidogrel sulfate was purchased from TCIchemicals (Portland, OR). Adenosine diphosphate (ADP) was purchased from Chrono-log Corporation (Havertown, PA). NS309, phenylephrine, uridine triphosphate (UTP), Drabkin's reagent, and human hemoglobin were purchased from Millipore Sigma (Burlington, MA).

2.2 Animal Care and Use

The procedures used in this study were approved by the Institutional Animal Care and Use Committee (protocol number 202000209). They were performed by following Michigan State University guidelines and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Michigan State University Campus Animal Resources provided all veterinary care.

2.3 $P2Y_{12}^{-/-}$ Mice

Wild-type, $P2Y_{12}^{+/-}$, and $P2Y_{12}^{-/-}$ mice (16-18 weeks; n=107) were bred in-house on a C57/BL6N background. Cryopreserved P2ry12 KO sperm (C57BL/6N-*P2ry12tm1*$^{(KOMP)Vlcg}$/Mmucd, RRID: MMRRC_048937-UCD) was imported from Mutant Mouse Resource and Research Centers and revived by *in vitro* fertilization following JAX protocol, using C57BL/6N females as egg donors [16]. Wild-type C57/BL6N (n=15) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Mice had free access to standard

chow and fresh water and were maintained on an automated 12/12-hour light/dark cycle with 7:00 am as the start of the light phase.

2.4 Drug Administration

Drugs were suspended in Nutella. Vehicle and 10 mg/kg clopidogrel were given to mice every day for 5 days. The doses of clopidogrel used have been demonstrated to inhibit platelet aggregation. On the day of the study, 24 hours after the 5th and final dose, mice were anesthetized with 3% isoflurane. Mice were used for blood loss measurements, platelet activation, cerebral blood flow measurements, and pressure myography as described below.

2.5 Blood Loss Measurements

Once mice were anesthetized, the tail was amputated 5 mm from the tip using a new number 10 surgical blade. The mouse was then immediately placed in a horizontal position, and the tail was immersed in 1.5 ml of Drabkin's reagent for 30 minutes [16]. A standard hemoglobin curve was generated using human hemoglobin with known concentrations. Hemoglobin concentrations from tail blood loss were measured on a Tecan Infinite M1000 Pro Microplate Reader at 540 nm. Data were analyzed using iControl software and then normalized to the human hemoglobin curve.

2.6 Platelet Activation

Once mice were anesthetized, blood was collected via cardiac puncture into a syringe containing heparin as an anticoagulant (1:10 150 Units/mL heparin to blood ratio). Platelets were identified using anti-CD42d binding, gated based on size and doublet discrimination, and then platelet activation was determined by Jon/A binding in whole blood stimulated by ADP. Heparinized blood (50 µL) was washed with 200 µL Tris-Hep buffer and with 1 mL Tyrode's

buffer. Blood was spun down at 900 rpm for 5 minutes. The pellet was resuspended in 1.25 mL Tyrode's buffer, and $CaCl_2$ (1 mM) was added. The washed blood (25 µL) was then incubated with ADP (20 µM), or Tyrode's buffer for 2 minutes. Jon-A (PE) and anti-CD42d (APC) were then added to these samples and incubated for 15 minutes in the dark. Flow cytometric assessment was performed using a BD Accuri C6 (BD Biosciences) available in the MSU South Campus Flow Cytometry Core Facility. Events (20,000) were collected on a log scale for FSC-A and then gated on the CD42d platelet scatter-based population followed by doublet discrimination. Jon-A positive platelets were quantified as a measure of platelet activation. Data were analyzed using CFlow Plus software, v1.0.227.04 (BD Biosciences).

2.7 Cerebral Blood Flow

Pial artery blood flow was measured by laser speckle contrast imaging (PeriMed Instruments). After anesthesia, a midline incision was made in the skin and the skull was exposed and cleaned. Two alternating layers of clear shiny and matte nail polish were applied to the skull to improve the image obtained by the laser. The laser speckle was positioned 12-13 cm above the skull and the isoflurane concentration was reduced to 1.5% for 3 minutes before imaging to decrease the vasodilation artifact caused by isoflurane. Regions of interest measurements of frontal, parietal, and temporal regions were drawn as previously described [17]. Mean perfusion was calculated in these regions for both hemispheres.

2.8 Pressure Myography

After blood collection, the mice were euthanized by decapitation. The brain was harvested and placed in cold Ca^{2+}-free physiological salt solution (PSS) containing 141.9 mM NaCl, 4.79 mM KCl, 1.12 mM KH_2PO_4, 1.79 mM $MgSO_4 \bullet 7H_2O$, 109 mM HEPES, and 59 mM dextrose. The left and right PCAs were then dissected from the brain and cleaned of any connective tissue. Each PCA was cannulated on 120 µm glass cannulas in a custom-made cannulation chamber.

The arteries were pressurized to an intraluminal pressure of 60 mmHg. A leak test was performed which encompassed pinching the tubing to observe if there was any decrease in the inner diameter. Only PCAs that held pressure were used for studies. The intraluminal pressure was then returned to 20 mmHg and Ca^{2+}-containing bicarbonate buffer containing 124 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 1 mM NaH_2PO_4, 4 mM dextrose, 23 mM $NaHCO_3$, and 2 mM $CaCl_2$ was added to the circulating bath and warmed to 37° C. The PCA was then pressurized to 60 mmHg at which point myogenic tone developed. Once the lumen diameter stabilized, increasing concentrations of UTP (10^{-9}–10^{-5} M) were added to the bath to assess vasoconstriction. The lumen diameter was measured using a 4X objective (Nikon Plan objective; Numerical Aperture: 0.25) with a Leica Leitz DM IL microscope. The average lumen diameter at each agonist concentration was recorded using VasoTracker software [18]. At the end of the experiment, ethylene glycol- bis(β-aminoethyl)-N, N, N', N'-tetraacetic acid (EGTA; 2 mM) and sodium nitroprusside (SNP; 10 µM) in Ca^{2+}-Free PSS were added to the bath to obtain a measure of the maximum passive diameter of each PCA.

The biomechanical properties of the PCA were assessed after dilation to Ca^{2+}-Free PSS/EGTA/SNP stabilized. A pressure-response curve was constructed by increasing the intraluminal pressure by 20 mmHg increments, starting from 0 mmHg and ending at 120 mmHg. The PCAs were equilibrated at each pressure for 5 minutes and the lumen and outer diameters were measured. The outer and inner diameters were then used to calculate distensibility and arterial stiffness as previously described [19,20].

2.9 Removal of Endothelium

The PCA was isolated from mice as described in subsection 2.7. To remove the endothelium, an air bubble was passed through the lumen followed by rubbing the lumen of each vessel with a silk suture before cannulation. The PCA was then pressurized to 60 mmHg at 37° C with Ca^{2+}-containing bicarbonate buffer until the generation of myogenic tone. Once the PCA lumen diameter stabilized, increasing concentrations of UTP were added to the vessels (10^{-}

^{9}–10^{-5} M) to test vasoconstriction. Finally, 10^{-6} M NS309 was added to the bath to test for complete denudation of the endothelium. The NS309 response in endothelium removed PCAs was <20% dilation. If PCAs dilated more than 20% they were removed from the study. At the end of the experiment, Ca^{2+}-Free PSS containing EGTA and SNP were added to the bath to obtain a measure of the maximum dilation achievable by each PCA.

2.10 Statistical Analysis

Data were analyzed using GraphPad Prism version 9.0. Analyses of platelet activation, hemoglobin concentration, and cerebral blood flow were performed by one-way analysis of variance (ANOVA) and compared to the vehicle using Tukey's post-test. Pressure response curves and constriction responses were analyzed by two-way ANOVA and compared to the vehicle using the Šídák post hoc test. In all cases, results were considered significant at $p<0.05$. Significance was denoted as *$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$.

3. Results

3.1 Clopidogrel-Treated Mice and $P2Y_{12}^{-/-}$ Mice have Increased Blood Loss

I studied the effects of blood loss in $P2Y_{12}^{+/-}$ and $P2Y_{12}^{-/-}$ mice and compared their effects to wild-type mice (Figure 21). Blood loss was collected for 30 minutes after 5 mm tail amputation in Drabkin's reagent and the hemoglobin concentration was measured. $P2Y_{12}^{-/-}$ mice had increased hemoglobin concentration compared to wild-type mice. $P2Y_{12}^{+/-}$ mice had an intermediate response. This verifies that the loss of $P2Y_{12}$ increases blood loss.

I also examined the effects of clopidogrel treatment on blood loss in wild-type, $P2Y_{12}^{+/-}$, and $P2Y_{12}^{-/-}$ mice (Figure 21). Wild-type mice treated with 10 mg/kg clopidogrel have increased hemoglobin concentration compared to wild-type vehicle-treated mice. $P2Y_{12}^{-/-}$ mice treated with 10 mg/kg clopidogrel did not have increased hemoglobin concentration compared to $P2Y_{12}^{-/-}$ vehicle-treated mice. However, the hemoglobin concentration may potentially be

maxed out in the $P2Y_{12}^{-/-}$ mice, making it impossible to observe additive bleeding by clopidogrel treatment. This data suggests that clopidogrel treatment and the loss of $P2Y_{12}$ increase blood loss.

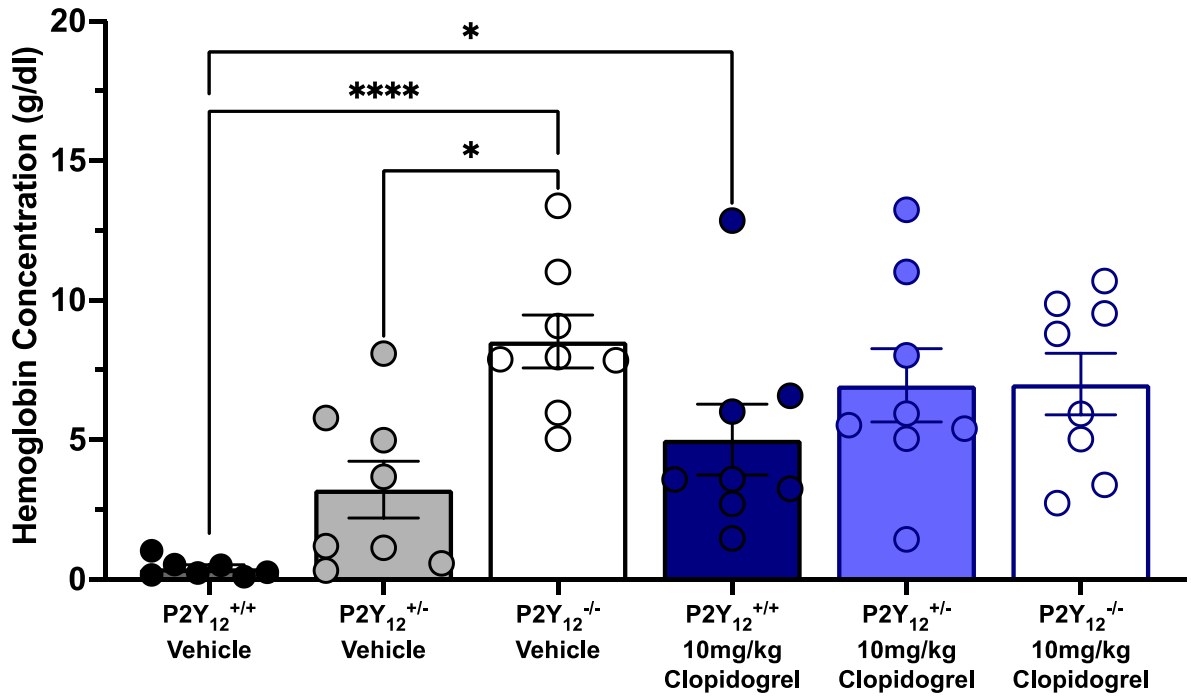

Figure 21: Blood loss in $P2Y_{12}^{-/-}$ mice. Blood loss hemoglobin concentrations in wild-type, $P2Y_{12}^{+/-}$, and $P2Y_{12}^{-/-}$ mice after 5-day treatment with vehicle or 10 mg/kg clopidogrel. Blood was collected in Drabkin's reagent for 30 minutes and hemoglobin concentration was measured. The data is presented as the mean of eight mice ± SEM. *p<0.05, ****p<0.0001 by one-way ANOVA followed by Tukey's multiple comparison test.

3.2 $P2Y_{12}^{-/-}$ Mice and Clopidogrel-Treated Mice have Reduced ADP Platelet Activation

The effects of $P2Y_{12}$ knockout and the $P2Y_{12}$ antagonist, clopidogrel, on ADP- and U46619-induced platelet activation were measured by flow cytometry (Figure 22 and 23, respectively). The CD42d antibody labels platelets through recognition of platelet glycoprotein V. The Jon/A antibody detects the formation of integrin $\alpha_{IIb}\beta_3$ on platelets.

$P2Y_{12}^{+/-}$ vehicle-treated and $P2Y_{12}^{-/-}$ vehicle-treated mice had decreased ADP-induced platelet activation when compared to wild-type vehicle-treated mice (Figure 22). Additionally, $P2Y_{12}^{-/-}$ vehicle-treated mice had decreased platelet activation when compared to $P2Y_{12}^{+/-}$ vehicle-

treated mice. Clopidogrel treatment decreased platelet activation in wild-type mice. Clopidogrel treatment did not further decrease platelet activation in $P2Y_{12}^{+/-}$ and $P2Y_{12}^{-/-}$ mice. These results verify that loss of $P2Y_{12}$ as well as inhibition of $P2Y_{12}$, by clopidogrel, inhibit platelet activation. Furthermore, this indicates that 10 mg/kg clopidogrel treatment replicates the decreased ADP-induced platelet activation that is observed with the loss of $P2Y_{12}$.

U46619-induced platelet activation was unchanged among treatment groups (Figure 23). This data indicates the inhibition of $P2Y_{12}$ with clopidogrel treatment and the genetic loss of $P2Y_{12}$ did not impair an alternative platelet activation mechanism. Therefore, indicating that the function of the platelets is still intact.

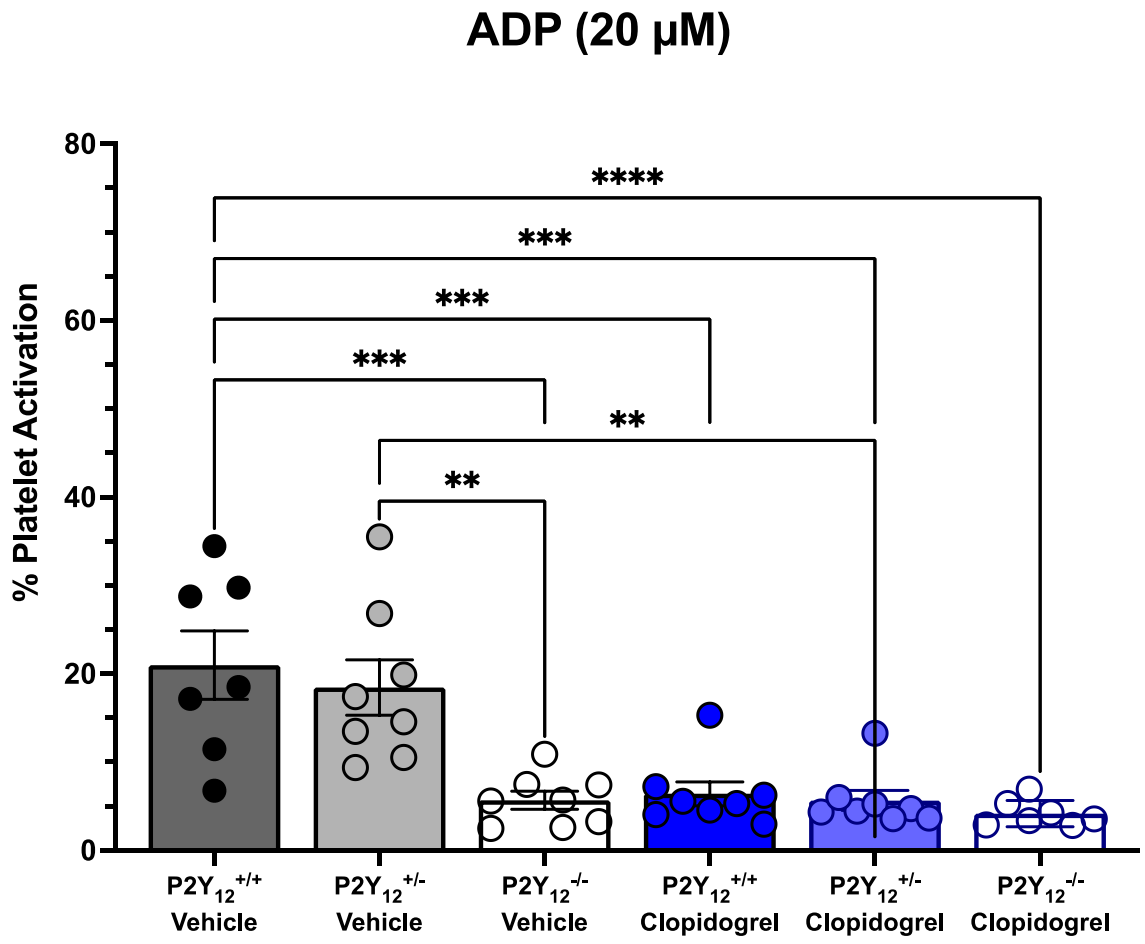

Figure 22: ADP-induced platelet activation in $P2Y_{12}^{-/-}$ mice. Flow cytometric assessment of platelets activated with 20 µM ADP after 5-day treatment with vehicle or 10 mg/kg clopidogrel in wild-type, $P2Y_{12}^{+/-}$, and $P2Y_{12}^{-/-}$ mice. The data represent the mean of eight mice ± SEM. **p<0.01, ****p<0.0001 by one-way ANOVA followed by Tukey's multiple comparison test.

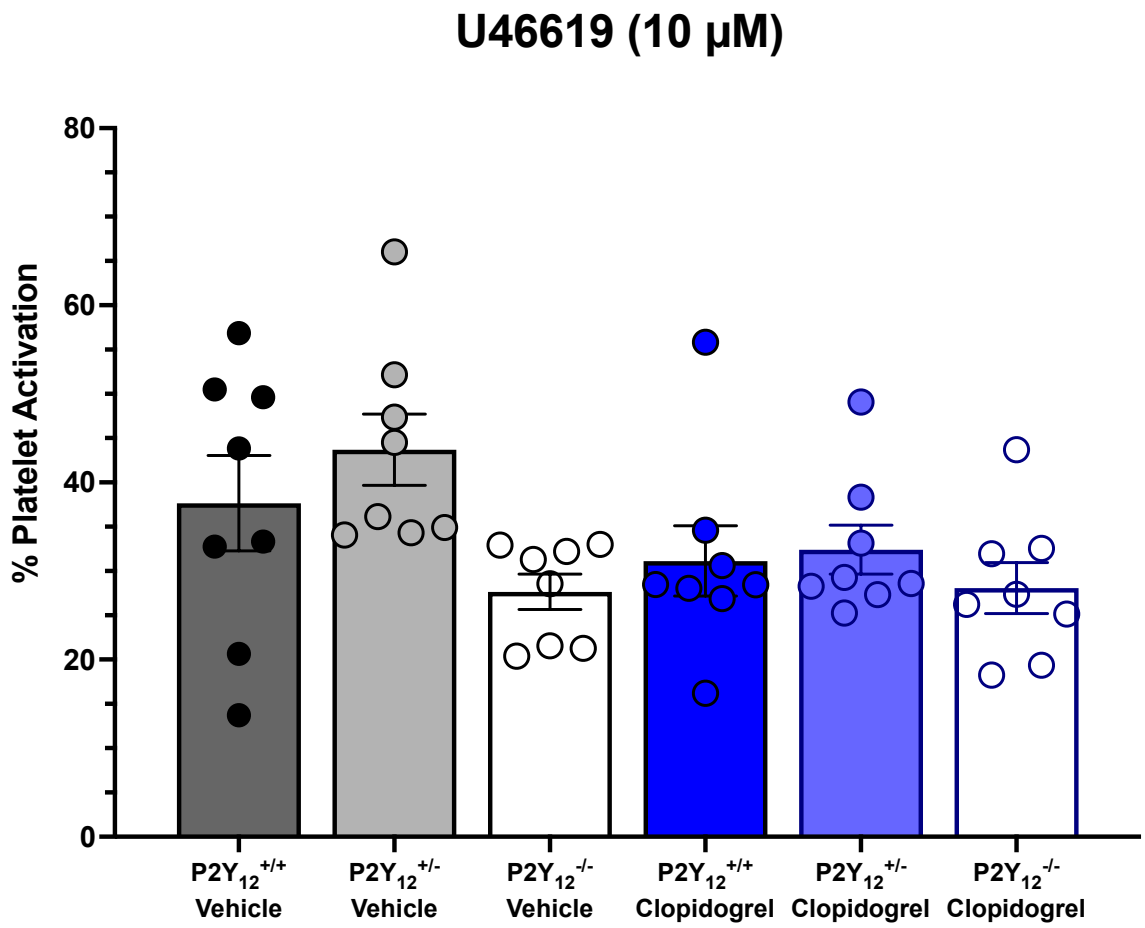

Figure 23: U46619-induced platelet activation in $P2Y_{12}^{-/-}$ mice. Flow cytometric assessment of platelets activated with 10 µM U46619 after 5-day treatment with vehicle or 10 mg/kg clopidogrel in wild-type, $P2Y_{12}^{+/-}$, and $P2Y_{12}^{-/-}$ mice. The data represent the mean of eight mice ± SEM.

3.3 Cerebral Blood Flow is not Altered in $P2Y_{12}^{-/-}$ Mice or Clopidogrel-Treated Mice

I determined the effect of $P2Y_{12}$ receptor knockout and clopidogrel treatment on cerebral blood flow (Figure 24). The loss of $P2Y_{12}$ does not change cerebral perfusion in the frontal, parietal, temporal, or total regions. Additionally, treatment with clopidogrel did not change cerebral blood flow. These results suggest that the $P2Y_{12}$ receptor is not important for baseline cerebral blood flow and that clopidogrel treatment has no effect.

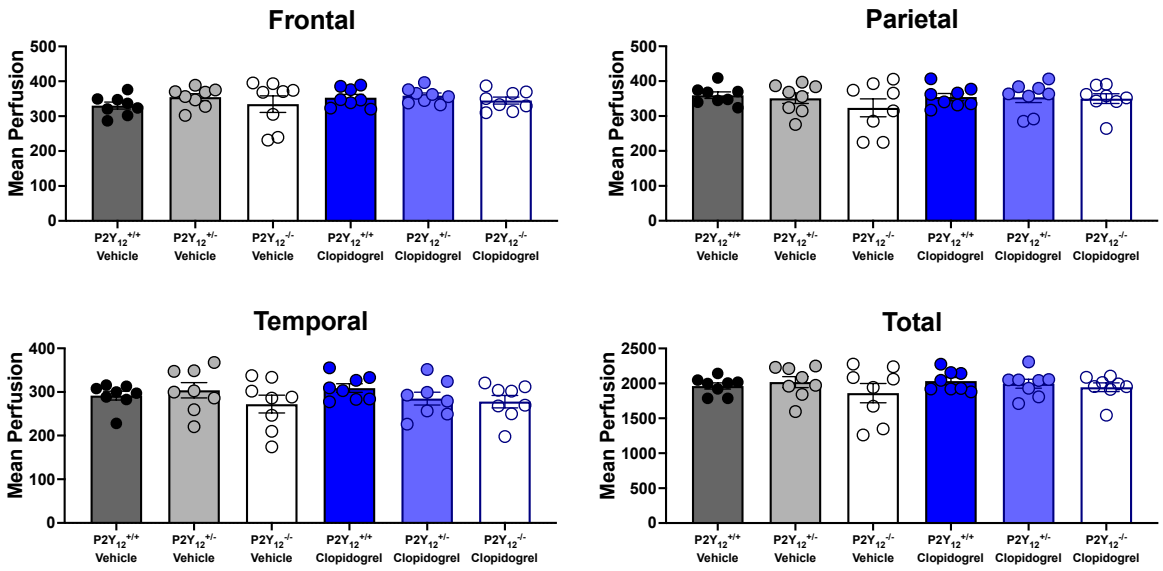

Figure 24: Cerebral blood flow in P2Y$_{12}^{-/-}$ mice. Cerebral blood flow was measured before euthanasia in anesthetized mice by laser speckle imaging. A) frontal region, B) parietal region, C) temporal region, and D) a total of all regions. The data are presented as the mean of eight mice ± SEM.

3.4 The Biomechanical Properties of Posterior Cerebral Arteries from P2Y$_{12}^{-/-}$ Mice and Clopidogrel-Treated Mice are Altered

I examined the biomechanical properties of PCAs from wild-type, P2Y$_{12}^{+/-}$, and P2Y$_{12}^{-/-}$ mice (Figure 25). The lumen diameter and outer diameter of PCAs from P2Y$_{12}^{+/-}$ and P2Y$_{12}^{-/-}$ mice were unchanged compared to wild-type mice (Figures 25A and 25B). PCAs from P2Y$_{12}^{-/-}$ mice have reduced distensibility, thereby signifying an impairment in the ability of the PCA wall to stretch passively with changes in pressure (Figure 25C). Furthermore, PCAs from P2Y$_{12}^{-/-}$ mice have increased arterial stiffness (Figure 25D). This indicates that P2Y$_{12}^{+/-}$ mice do not have altered PCA structure, but PCAs from P2Y$_{12}^{-/-}$ mice have reduced distensibility and increased arterial stiffness.

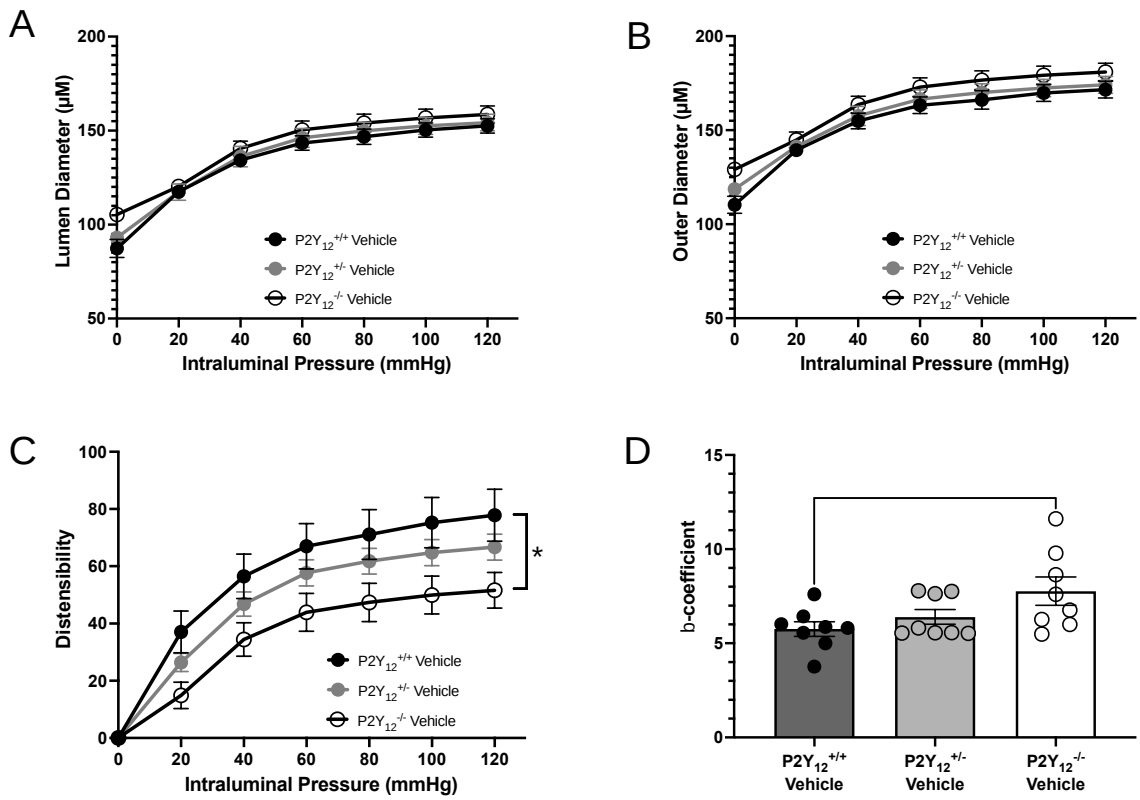

Figure 25: P2Y$_{12}^{-/-}$ mice have decreased distensibility and increased arterial stiffness. The biomechanical properties were assessed in isolated posterior cerebral arteries from vehicle-treated mice using pressure myography. A) lumen diameter, B) outer diameter, C) distensibility, and D) β-coefficient. The data are presented as mean ± SEM. Distensibility is compared by two-way ANOVA with repeated measures followed by the Šídák post hoc test. β-coefficient is compared by one-way ANOVA followed by Dunnett's post hoc test. *p<0.05, n=8/group.

I next examined the structure of PCAs from wild-type and P2Y$_{12}^{-/-}$ mice treated with clopidogrel (Figure 26). The lumen and outer diameter of PCAs were unchanged with clopidogrel treatment (Figures 26A and 26B). However, clopidogrel treatment decreased distensibility (Figure 26C) and increased arterial stiffness (Figure 26D). Interestingly, the biomechanical properties of PCAs from clopidogrel-treated P2Y$_{12}^{-/-}$ mice were not changed. This data demonstrates clopidogrel treatment impairs PCAs in wild-type mice, and that clopidogrel treatment in the absence of P2Y$_{12}$ has protective effects on the vasculature.

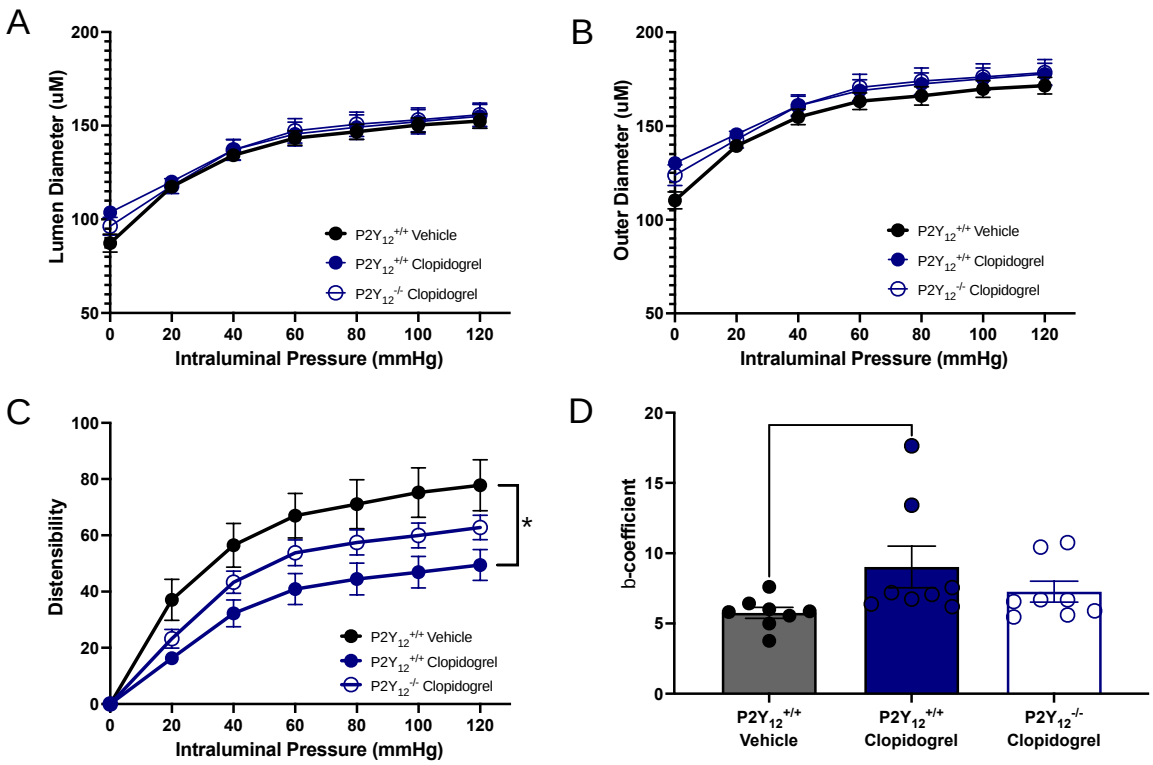

Figure 26: Clopidogrel treatment decreases distensibility and increases arterial stiffness. The biomechanical properties were assessed in isolated posterior cerebral arteries from vehicle- and clopidogrel-treated mice using pressure myography. A) lumen diameter, B) outer diameter, C) distensibility, and D) β-coefficient. The data are presented as mean ± SEM. Distensibility is compared by two-way ANOVA with repeated measures followed by the Šídák post hoc test. β-coefficient is compared by one-way ANOVA followed by Dunnett's post hoc test. *p<0.05, n=8/group.

3.5 Response of UTP Vasoconstriction in Posterior Cerebral Arteries in P2Y$_{12}^{-/-}$ Clopidogrel-Treated Mice

PCAs were allowed to generate myogenic tone before stimulation to UTP. The PCAs that did not generate at least 12% myogenic tone were preconstricted with 3 µM phenylephrine. There was no significant difference in total constriction between posterior cerebral arteries that generated myogenic tone compared to preconstricted arteries (Figure 27). PCA vascular reactivity was assessed by adding UTP (P2Y$_2$ and P2Y$_4$ selective agonist) to the bath (Figure 28). UTP stimulates concentration-dependent vasoconstriction in the PCA. Clopidogrel treatment and the loss of P2Y$_{12}$ did not impair UTP-mediated vasoconstriction.

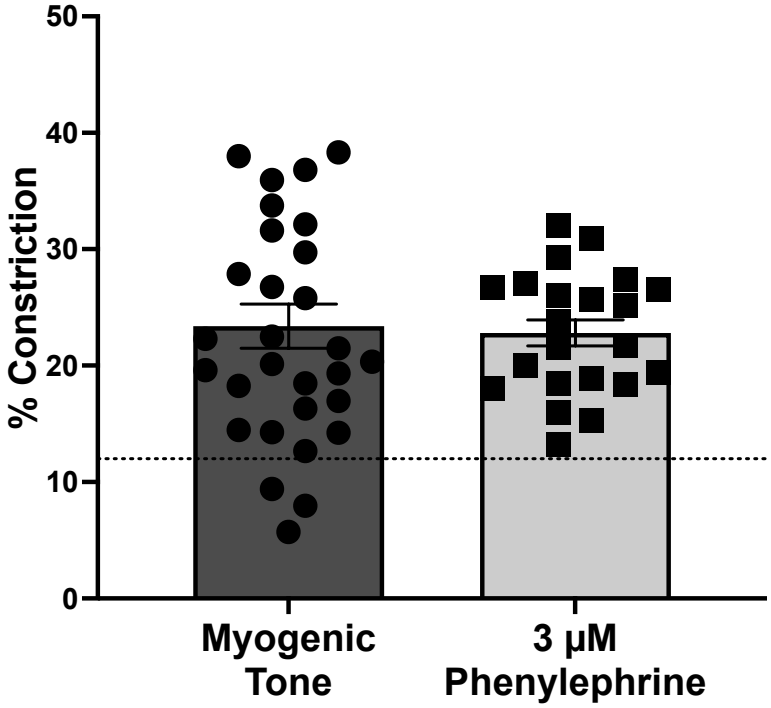

Figure 27: Percent constriction of the myogenic tone or phenylephrine response. Percent constriction was unchanged between myogenic tone and phenylephrine (3 µM) groups. The data are presented as the mean ± SEM. Myogenic tone: n=30, phenylephrine: n=22.

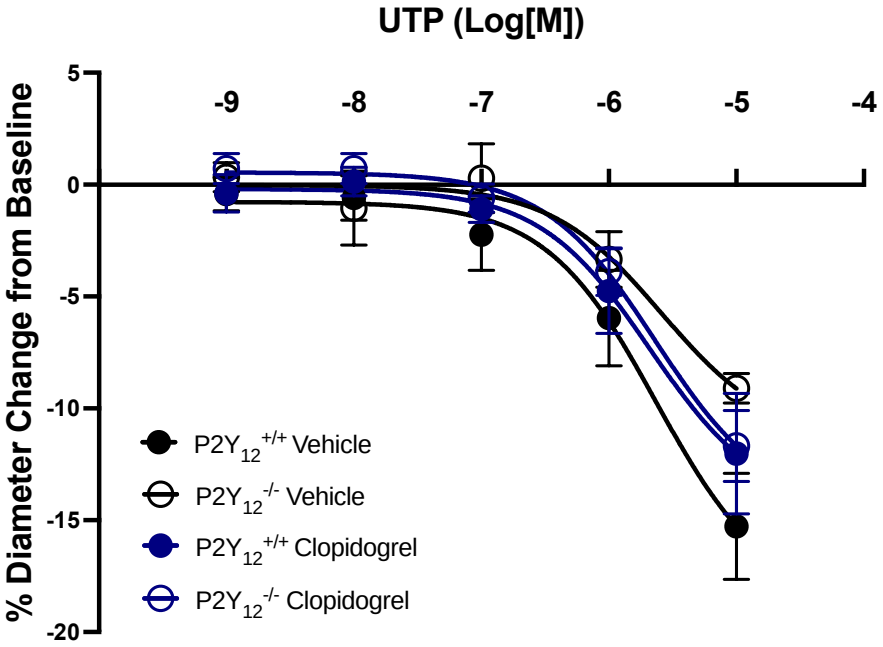

Figure 28: UTP constriction in the posterior cerebral artery. UTP-mediated vasoconstriction in the posterior cerebral artery after pretreatment with vehicle or 10 mg/kg clopidogrel in wild-type and $P2Y_{12}^{-/-}$ mice. The data are presented as the mean ± SEM. n=6/group.

I next used endothelium-denuded PCAs to determine if UTP vasoconstriction was endothelium-dependent. This study was performed to determine the location of the $P2Y_2$ and $P2Y_4$ receptors in PCAs from mice. Removal of the endothelium did not alter the constriction response to UTP (Figure 29A). Additionally, the loss of $P2Y_{12}$ and clopidogrel treatment did not change UTP constriction in endothelium removed PCAs (Figure 29B). This suggests that $P2Y_2$ and $P2Y_4$ receptor activation is not dependent on the endothelium.

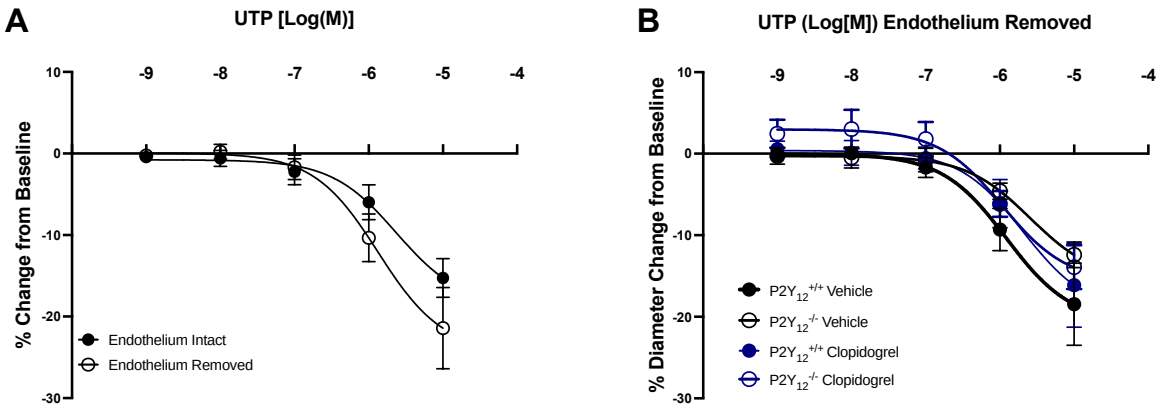

Figure 29: UTP constriction in endothelium removed posterior cerebral arteries. A) UTP-mediated vasoconstriction in the posterior cerebral arteries with intact or removed endothelium from wild-type vehicle-treated mice. B) UTP-mediated vasoconstriction in the posterior cerebral artery after pretreatment with vehicle or 10 mg/kg clopidogrel in wild-type and $P2Y_{12}^{-/-}$ mice in vessels with endothelium removed or intact. The data are presented as the mean ± SEM. n=6/group.

4. Discussion

The novel findings in this study are that: 1) cerebral blood flow is not altered in $P2Y_{12}$ deficient mice or clopidogrel-treated mice, 2) PCAs from $P2Y_{12}^{-/-}$ mice and clopidogrel-treated mice have increased arterial stiffness and decreased distensibility, and 3) clopidogrel treatment in $P2Y_{12}^{-/-}$ mice attenuated the increased arterial stiffness and decreased distensibility observed in vehicle-treated $P2Y_{12}^{-/-}$ mice. Taken together, our data suggest clopidogrel treatment has similar effects on blood loss, platelet activation, the biomechanical properties of the vessel, and vascular function compared to the loss of $P2Y_{12}$. I propose that the loss of $P2Y_{12}$,

genetically or pharmacologically, impairs the biomechanical properties of the PCA which could potentiate the bleeding in at-risk individuals.

The hemoglobin measurements demonstrate an increase in bleeding in both clopidogrel-treated mice and $P2Y_{12}$ deficient mice (Figure 21). A previous study by André et al. found an increase in bleeding in clopidogrel-treated $P2Y_{12}^{-/-}$ mice [21]. Interestingly, my results with 10 mg/kg clopidogrel-treated $P2Y_{12}^{-/-}$ mice did not reproduce this observation. This can be rationalized by the differences in methodology between the studies; André et al. used 50 mg/kg clopidogrel, 2 mm tail amputation, 2 hours post drug administration, and 15-minute bleeding duration, while I used 10 mg/kg clopidogrel, 5 mm tail amputation, 5 repeated doses, 24 hours post last dose, and 30-minute bleeding duration. In blood loss studies, each factor can produce a difference in the results.

The platelet activation studies confirmed clopidogrel treatment and $P2Y_{12}$ deficiency inhibit ADP-induced activation (Figure 22). I also verified that the platelets are still functional through the activation by U46619, an alternative platelet activation mechanism (Figure 23).

The risk of intracerebral hemorrhage increases with patients on antiplatelet therapies, specifically clopidogrel [6,9,11]. This is typically due to the decrease in platelet activation at the site of a cerebral microbleed [10,22]. However, one characteristic of clopidogrel antiplatelet therapy that increases the risk further is the inhibition of vasoconstriction [15]. The first step in hemostasis is vasoconstriction, followed by platelet activation. Cerebral microbleeds need both of those mechanisms to prevent bleeding. Since clopidogrel treatment inhibits both vasoconstriction and platelet activation there could be a further increase in blood loss at the site of damage. This impairment in hemostasis could shift the cerebral microbleed to a detrimental intracerebral hemorrhage.

The pial arteries regulate pressure and blood flow to the cerebral circulation and are commonly assessed for cerebral perfusion [23,24]. My results demonstrate that cerebral blood flow is unchanged in both clopidogrel-treated mice and $P2Y_{12}$ deficient mice (Figure 24). The PCAs regulate pressure and blood flow to the posterior cerebral circulation. This artery is frequently

chosen to study as it is preferred for pressure myography over the middle cerebral artery, which is highly branched in mice. PCAs have also been reported to be a site associated with intracerebral hemorrhage making them an ideal choice for this study [25,26]. The key observation from our study demonstrated that the knockdown of $P2Y_{12}$, genetically or pharmacologically, is associated with changes in the biomechanical properties of the PCAs. PCAs from $P2Y_{12}^{-/-}$ mice and clopidogrel-treated mice had increased arterial stiffness and decreased distensibility (Figures 25 and 26). Stiffened arteries cannot stretch when faced with increased pressure [27,28]. Cerebral microbleeds are vulnerable to these increases in pressure thereby making them more susceptible to intracerebral hemorrhage. I did not measure blood pressure and heart rate in these mice, however stiffer arteries with reduced distensibility are associated with cerebral microbleeds and intracerebral hemorrhage [29–32]. Decreased arterial distensibility is also associated with ruptured intracranial aneurysms [33]. The modification of arterial wall properties that are important in determining stiffness, such as elastin and collagen deposition, can increase the risk of vascular rupture in the face of pressure changes [32–34]. Taken together, this suggests the changes to vascular structure associated with clopidogrel treatment could be an additional mechanism by which clopidogrel causes bleeding.

My previous findings on clopidogrel's ability to inhibit $P2Y_2$-mediated vasoconstriction indicate that clopidogrel-treated arteries do not constrict adequately in response to vascular injury thereby causing excessive bleeding. One goal of this study was to replicate those findings in $P2Y_{12}$ deficient animals to fully elucidate the mechanistic action of $P2Y_2$ inhibition. Surprisingly, we did not observe a decrease in UTP-mediated vasoconstriction in clopidogrel-treated mice or $P2Y_{12}^{-/-}$ mice (Figure 28). The expression of purinergic receptors is different between vessels and species. Mice may not have $P2Y_2$ receptors in the PCAs. Therefore, UTP may only be activating $P2Y_4$ to induce vasoconstriction. The expression of P2Y receptors in the mouse PCAs must be further examined.

One limitation of this study is the use of PCA instead of the middle cerebral artery. My previous study on $P2Y_2$ inhibition was done in the middle cerebral artery of rabbits. However, to investigate the effects observed in $P2Y_{12}^{-/-}$ animals we switched to a mouse model. The middle cerebral artery in a mouse is extensively branched, making it problematic to study. We used the PCA as it is still implicated in cerebrovascular events, particularly intracerebral hemorrhage [25,26]. The specific location where $P2Y_{12}$, $P2Y_2$, and $P2Y_4$ are expressed in the PCA of mice is uncertain. My vascular function data suggests these receptors are on the VSMCs, as the response was unchanged in endothelium-removed PCAs (Figure 29). However, the expression of the specific receptors needs to be quantified through RT-PCR in subsequent studies.

In conclusion, blood loss was increased in clopidogrel-treated and $P2Y_{12}$-deficient mice. Additionally, platelet activation was inhibited in $P2Y_{12}$-deficient mice and clopidogrel-treated mice. Further analysis of the adverse effects of the loss of $P2Y_{12}$ included increased arterial stiffness and decreased distensibility, which were also observed with clopidogrel-treated mice. Our results suggest that loss of $P2Y_{12}$, either genetically or pharmacologically, impairs the biomechanical properties of the PCAs, which could explain the adverse bleeding side effects observed with clopidogrel treatment. The mechanism by which the biomechanical properties of the PCAs are altered by clopidogrel treatment and the knockout of $P2Y_{12}$ must be further studied.

Acknowledgments

I would like to thank Matt Bernard for his help in the development of the Flow Cytometry assay. I would also like to thank Huirong Xie and Elena Demireva for their help in generating the $P2Y_{12}^{-/-}$ founders for our breeding colony. Additionally, I would like to thank Nathan Tykocki for his proofreading and helpful suggestions.

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CHAPTER 5

Summary, Novel Findings, Limitations, Future Directions, and Final Perspectives

1. Summary

The mechanism by which clopidogrel causes adverse bleeding was presumed to be through platelet $P2Y_{12}$ inhibition. The additional 16 metabolites of clopidogrel were deemed inactive. However, patients who cannot form the M13 metabolite, due to a lack of functional CYP2C19, have a higher rate of thrombotic events but a similar rate of bleeding compared to patients with normal CYP2C19 function also on clopidogrel [1]. These data suggest that the prodrug clopidogrel or one of the other metabolites generated via CYP2C19-independent pathways are responsible for adverse bleeding. The mechanism by which clopidogrel potentiates bleeding must be determined as more than 30% of patients on clopidogrel have cerebral microbleeds after one year [2–5]. This presence of cerebral microbleeds increases the risk of intracerebral hemorrhage [6,7].

Further evidence of off-target bleeding effects with clopidogrel was demonstrated by André et al. They observed that clopidogrel-treated $P2Y_{12}^{-/-}$ mice had significantly increased bleeding compared to vehicle-treated $P2Y_{12}^{-/-}$ mice [8]. They were one of the first to report non-$P2Y_{12}$ mediated bleeding associated with clopidogrel treatment. Moreover, my study (see Chapter 1, Part 2) provided evidence that the adverse bleeding associated with clopidogrel was not due to the M13 metabolite [9]. Therefore, I wanted to determine how clopidogrel induced bleeding since previous reports suggest it is not due to $P2Y_{12}$ inhibition or by the M13 metabolite [8,9].

The data presented in this dissertation reveal that clopidogrel given *in vivo* is more than just a $P2Y_{12}$ antagonist. My studies support the overall hypothesis that clopidogrel treatment affects the vasculature which results in adverse bleeding. In summary, I determined clopidogrel inhibits endothelial $P2Y_2$ receptor vasoconstriction in the middle cerebral artery (MCA) from rabbits in Chapter 2. Next, I determined that $P2Y_2$ is not inhibited by the prodrug clopidogrel, M1, M2, or M13 metabolites in Chapter 3. Then in Chapter 4, I observed changes to the biomechanical properties of the posterior cerebral artery (PCA) in mice after clopidogrel treatment. My goal was to characterize the off-target effects of clopidogrel and determine how

those effects promote bleeding in the cerebral vasculature. This was tested through three specific aims.

The first aim was designed to assess the inhibition of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and/or $P2Y_{14}$ by clopidogrel treatment. I choose to assess all P2Y receptors in this study because of the structural similarity of the receptors, as well as the chemical similarity between the clopidogrel metabolites [10]. I was not able to evaluate $P2Y_{13}$ because a selective agonist is not available for this receptor. For this aim, I used a pharmacological approach with $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, and $P2Y_{14}$ selective agonists in the MCAs of rabbits. Rabbits were treated orally for 3 days with vehicle or clopidogrel. On the 4^{th} day, the brains were collected and the MCAs were mounted in a pressure myography system. MCAs were chosen to study as they are the artery most pathologically affected in the brain [11]. Ruptured MCAs can lead to neurological disorders or death [12]. Unfortunately, patients on antithrombotic therapy, such as clopidogrel, are not suitable for surgery after ruptured MCAs [12]. Therefore, understanding the mechanism by which clopidogrel potentiates intracerebral hemorrhage, specifically in the MCA, is crucial.

Pressure myography was used in this study to assess MCA function. Pressure myography was chosen over wire myography for this aim as it mimics *in vivo* conditions [13]. It is an instrumental technique used to determine functional changes in the vasculature. For my study, increasing concentrations of the P2Y selective agonists were added through the lumen and the inner diameter was recorded. Changes in agonist-induced vasoconstriction were then observed.

The second aim was designed to determine which metabolite was responsible for $P2Y_2$ inhibition. At the time of this study, I was only able to obtain the clopidogrel prodrug, the M1 metabolite, and the M2 metabolite. For this aim, I used a pharmacological approach to observe inhibition caused by the clopidogrel prodrug, the M1 metabolite, and the M2 metabolite in 1321N1 astrocytoma cells stably transfected with $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$. Cells were loaded with a calcium dye, treated with the drug for 30 minutes, and then selective

agonists for each receptor were added to the plate. Calcium mobilization was measured and compared to vehicle treatment. The clopidogrel prodrug, the M1 metabolite, and the M2 metabolite were not further metabolized in this study. Astrocytoma cells do not endogenously express the CYP450s necessary for metabolism. Therefore, I know the response measured from these cells was only due to the metabolite and not downstream metabolites. The cell studies in this aim were designed to determine if the clopidogrel prodrug, the M1 metabolite, or the M2 metabolite inhibited any of the P2Y receptors.

Additionally, for the second aim, I determined platelet inhibition and blood loss in mice treated with clopidogrel, the M1 metabolite, and the M2 metabolite. Mice were treated orally for 5 days. On the 6th day, blood was collected for evaluation of platelet activation and blood loss measurements were taken. Since these drugs were given *in vivo*, further metabolism occurred. M1 is not further metabolized, but M2 is metabolized into 7 additional metabolites. One of the downstream metabolites of M2 is M13, therefore, I knew administration of M2 would inhibit platelet activation. If M1 increased bleeding, it would definitively suggest M1 is responsible for adverse bleeding. However, if M2 increased bleeding, it would narrow down the possible metabolite culprits to M2 or any metabolite downstream from it. The mouse studies in the second aim elucidated whether M1 and/or M2 were the metabolites responsible for adverse bleeding.

The third aim was designed to assess the adverse effects of clopidogrel treatment in the absence of the $P2Y_{12}$ receptor. The bleeding associated with clopidogrel use was assumed to be induced through the inhibition of $P2Y_{12}$. However, it is simple-minded to think that $P2Y_{12}$ inhibition is the only reason for clopidogrel bleeding since other $P2Y_{12}$ antagonists have been reported to not induce bleeding [14]. Therefore, I needed to demonstrate the adverse effects of clopidogrel treatment in $P2Y_{12}^{-/-}$ animals. Thus, for this aim, I used a genetic approach with $P2Y_{12}^{-/-}$ mice. Mice were treated with vehicle or clopidogrel for 5 days. On the 6th day, the brain was removed and the PCAs were cannulated in a pressure myography system. In mice, the MCA is highly branched making it nearly impossible to get a large enough segment to analyze

in a pressure myography system. Therefore, the next best cerebral artery was the PCA. The PCA is the terminal branch of the basilar artery and it is responsible for supplying blood to the occipital lobe, temporal lobe, thalamus, midbrain, and choroid plexus [15]. Intracerebral hemorrhages have been reported to arise in PCAs, validating them as an ideal cerebral artery to study for this aim [16,17]. Once the PCA was cannulated, they were pressurized from 0-120 mmHg, in 20 mmHg increments. The inner and outer diameters were recorded at each pressure and then used to calculate distensibility and arterial stiffness.

While the biomechanical vessel data was the most interesting in this aim, I also assessed platelet activation, blood loss, cerebral perfusion, and vascular function. Measuring platelet activation was crucial to validate that the dose of clopidogrel I used inhibited platelets. I measured blood loss to confirm that the loss of $P2Y_{12}$ and clopidogrel treatment increased bleeding. I also wanted to examine if clopidogrel treatment increased bleeding in the $P2Y_{12}^{-/-}$ mice. I next wanted to look at cerebral perfusion in these mice since stiffer arteries are associated with reduced blood flow [18,19]. Lastly, I wanted to replicate the $P2Y_2$ inhibition I observed in rabbit MCAs. Unfortunately, mouse PCAs are significantly smaller, making the addition of the $P2Y_2$ agonist to the lumen impossible. The flow-through rate of a mouse PCA was 10 µl/minute. Obtaining a concentration-response curve at this rate would take over 6 hours. At that point, the function of the PCA is unreliable. Therefore, $P2Y_2$ activation had to be via external stimulation. I added the $P2Y_2$-selective agonist I used in Chapter 2, MRS 2768, to the outside of the PCA and no vascular response was produced. As determined in Chapter 2 of this dissertation, MRS 2768 activation was endothelium-dependent. Therefore, I was unsure if $P2Y_2$ was not present in the PCAs or if MRS 2768 was not able to diffuse through the smooth muscle to activate the endothelium. The next best $P2Y_2$ agonist to examine was UTP. UTP activates both the $P2Y_2$ receptor and the $P2Y_4$ receptor. UTP did induce constriction in the mouse PCA. While UTP does not determine $P2Y_2$ constriction alone, it was the best choice available for this study. The studies in aim three examined the effects of clopidogrel treatment, the loss of $P2Y_{12}$, and clopidogrel treatment in $P2Y_{12}$ deficient animals.

2. Novel Findings

My studies show that the increase in bleeding caused by clopidogrel was not due to the active metabolite, M13. I am the first to demonstrate the inhibition of $P2Y_2$ by clopidogrel. Additionally, I determined clopidogrel impairs the biomechanical properties of the cerebral vasculature. The findings in this dissertation were as follows and are summarized in Figure 29:

2.1 Chapter 1, Part 2: DT-678 inhibits platelet activation with a lower tendency for bleeding compared to existing $P2Y_{12}$ antagonists

1. DT-678 (the M13 metabolite) did not significantly prolong bleeding time at doses capable of inhibiting platelet activation.
2. Clopidogrel significantly prolonged bleeding time without comparable platelet inhibition.

Taken together, these data suggest that the M13 metabolite is not responsible for the adverse bleeding associated with clopidogrel treatment. Since M13 is identified as the metabolite responsible for platelet inhibition, I can conclude from these studies that clopidogrel increases bleeding in a platelet-independent mechanism. This study also demonstrated that the administration of M13 alone provides equally effective antiplatelet properties albeit with a more favorable safety profile. The additional metabolites of clopidogrel are electrophilic which may nonselectively bind to circulating and cellular macromolecules. From this study, I proposed that an additional metabolite of clopidogrel impairs the vasculature to cause adverse bleeding.

2.2 Chapter 2: Clopidogrel Treatment Inhibits $P2Y_2$-Mediated Constriction in the Rabbit Middle Cerebral Artery

1. Clopidogrel treatment in rabbits did not significantly inhibit myogenic tone generation in MCAs but did reduce the percentage of MCAs that generated tone.

2. Clopidogrel treatment in rabbits significantly inhibited vasoconstriction resulting from $P2Y_2$ receptor activation.
3. $P2Y_2$-mediated vasoconstriction is endothelium-dependent in the rabbit MCA.

Taken together, this data suggests clopidogrel, or its metabolites, inhibit the endothelial $P2Y_2$ receptor in the cerebral vasculature. The $P2Y_2$ receptor was detected as the predominate P2Y subtype in isolated human endothelial cells via western blot and RT-PCR [20,21]. Endogenous UTP and ATP are released from vascular endothelial cells during shear stress and at the site of injury [22]. The release of UTP and ATP mediates vasoconstriction via P2Y receptors, like $P2Y_2$. Vasoconstriction is crucial in maintaining hemostasis since it reduces blood loss and aids in the formation of the platelet plug due to changing flow conditions [23–25]. The inhibition of $P2Y_2$ vasoconstriction provides a plausible explanation for which adverse cerebral bleeding events occur with clopidogrel treatment. Next, I hypothesized that one of the additional metabolites of clopidogrel was responsible for the $P2Y_2$ receptor inhibition.

2.3 Chapter 3: Evaluation of Clopidogrel Metabolites to Determine the Cause of Adverse Bleeding

1. The prodrug clopidogrel, the M1 metabolite, the M2 metabolite, and the M13 metabolite do not inhibit $P2Y_1$, $P2Y_2$, $P2Y_4$, or $P2Y_6$.
2. The prodrug clopidogrel, the M1 metabolite, and the M2 metabolite do not inhibit $P2Y_{12}$.
3. The M1 metabolite and the M2 metabolite do not cause an increase in blood loss.
4. The M1 metabolite does not inhibit platelet activation.

Taken together, these data suggest that the clopidogrel prodrug, M1, M2, and M13 metabolites are not responsible for the adverse bleeding associated with clopidogrel. The clopidogrel prodrug, the M1 metabolite, and the M2 metabolite did not inhibit any of the P2Y receptors in my cell-based study. This narrows down the possible metabolites responsible for $P2Y_2$ inhibition to the M3-M17 metabolites. To narrow this search further, I assessed the M2 metabolite in mice. This determined whether the bleeding was due to

metabolites downstream from M2 or outside of M2 metabolism. M2 did not increase hemoglobin concentrations, thereby indicating that M2 and its downstream metabolites, M11-M17, are not involved in the adverse bleeding associated with clopidogrel. The additional metabolites of clopidogrel, M3-M10, must be assessed individually to determine which metabolite is responsible for $P2Y_2$ inhibition and/or the increase in bleeding.

2.4 Chapter 4: Clopidogrel Treatment and Loss of $P2Y_{12}$ Impair the Biomechanical Properties of the Posterior Cerebral Arteries in Mice

1. $P2Y_{12}^{-/-}$ mice and clopidogrel-treated mice have increased blood loss.
2. PCAs from $P2Y_{12}^{-/-}$ mice and clopidogrel-treated mice have reduced distensibility and increased arterial stiffness.
3. UTP-mediated vasoconstriction was not inhibited in PCAs from $P2Y_{12}^{-/-}$ mice and clopidogrel-treated mice
4. UTP-mediated vasoconstriction was not endothelium-dependent.

Taken together, these data suggest that the loss of $P2Y_{12}$ activation, pharmacologically or genetically, impairs vascular structure. The studies in this chapter confirmed that the loss of $P2Y_{12}$, as well as clopidogrel treatment, impairs ADP-induced platelet activation. This inhibition was not potentiated in clopidogrel-treated $P2Y_{12}^{-/-}$ mice as maximum inhibition was already achieved in the $P2Y_{12}^{-/-}$ mice. I hypothesize this is what also occurred in my blood loss studies. It is harder to determine the maximum possible blood loss. However, because André et al. demonstrated increases in blood loss with clopidogrel treatment in $P2Y_{12}^{-/-}$ mice compared to vehicle treatment, at a less robust tail amputation, I believe the 5 mm tail amputation maxes out the blood loss findings. This would explain why I did not observe an increase in blood loss in clopidogrel-treated $P2Y_{12}^{-/-}$ mice compared to vehicle-treated $P2Y_{12}^{-/-}$ mice.

The cerebral perfusion data in this aim demonstrated that at this dose of clopidogrel and treatment timeframe, cerebral blood flow is not affected. It also suggests that $P2Y_{12}$ is not crucial in the maintenance of cerebral perfusion as it was unaltered in the $P2Y_{12}^{-/-}$ mice.

Lastly, UTP-mediated vasoconstriction was unchanged in clopidogrel-treated and $P2Y_{12}^{-/-}$ mice. This finding did not fully elucidate whether or not $P2Y_2$ was inhibited in these animals since $P2Y_4$ was also being activated. From my findings in Chapter 2, $P2Y_4$-mediated vasoconstriction was not impaired in clopidogrel-treated rabbits. The UTP-stimulated $P2Y_4$ response could be hiding the subtle changes in the UTP-mediated $P2Y_2$ response. $P2Y_2$ function must be further analyzed to determine inhibition by clopidogrel.

The changes in the biomechanical properties of the vessel observed in this chapter provide a new explanation as to how clopidogrel causes adverse bleeding clinically. Stiffer arteries with reduced distensibility are associated not only with cerebral microbleeds but also with intracerebral hemorrhage [26–30]. Stiffened arteries increase the pressure and pulsatile flow to the microvasculature in the brain. Pulsations generated by the heart are no longer mitigated in stiffened pial arteries [31]. This results in increased pressure and flow to the microvasculature, such as the parenchymal arterioles, putting them at risk for hemorrhage. Furthermore, arteries with reduced distensibility potentiate this risk as they have a reduced ability to expand and contract with the cardiac pulsations [32].

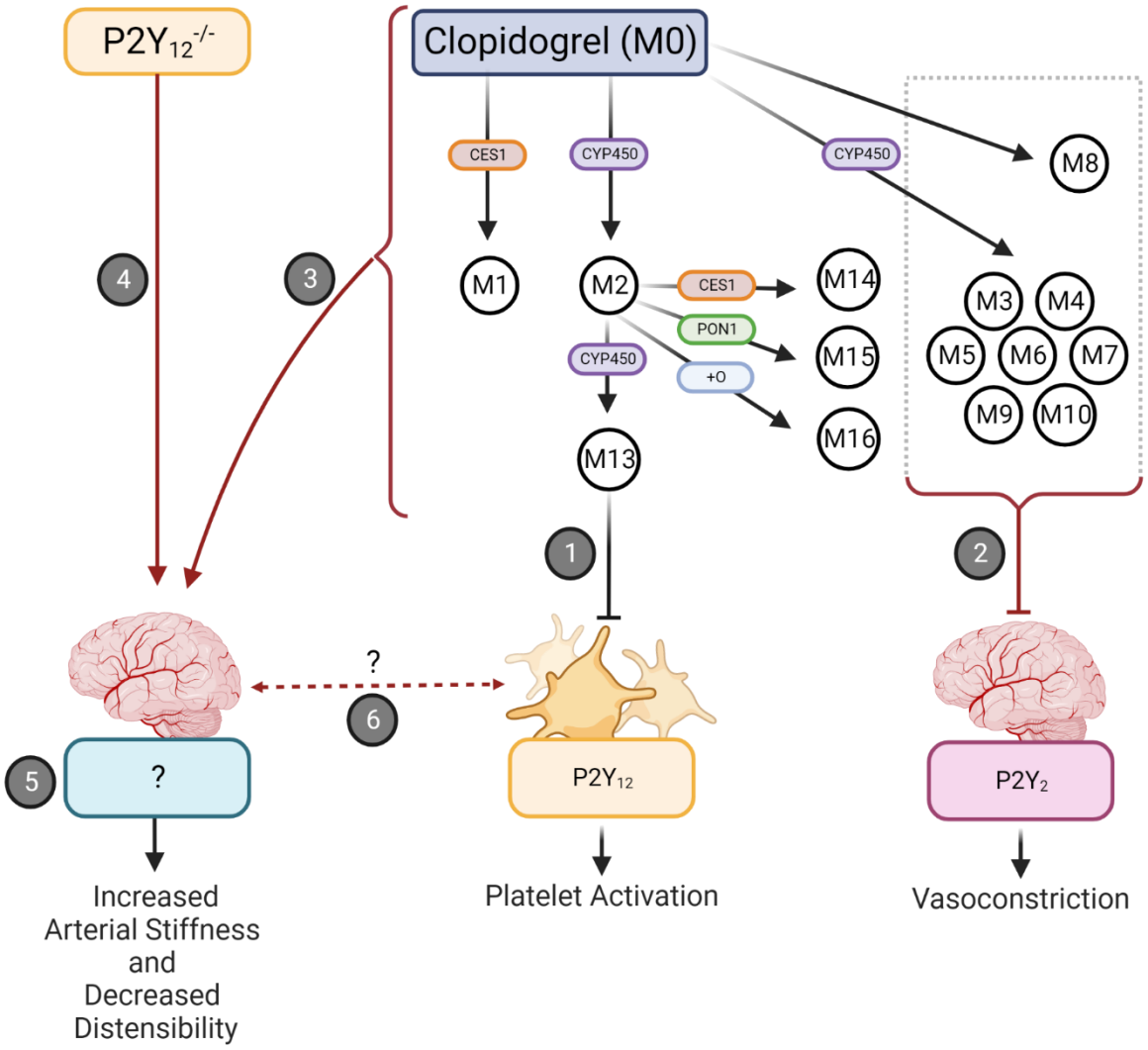

Figure 30: Summary of findings. (1) Clopidogrel treatment inhibits $P2Y_{12}$ activation on platelets. (2) Clopidogrel treatment inhibits endothelial $P2Y_2$-mediated vasoconstriction in the cerebral vasculature, but this is not due to the prodrug, the M1 metabolite, the M2 metabolite, or any metabolite downstream of M2. (3) Clopidogrel treatment increases arterial stiffness and decreases distensibility. (4) The loss of $P2Y_{12}$ also increases arterial stiffness and decreases distensibility. (5) The mechanism by which the biomechanical properties of the brain vasculature are altered is unknown. (6) Furthermore, it is uncertain whether the biomechanical properties are altered due to direct vascular mechanisms or the loss of platelet-vessel interaction.

3. Limitations

Some limitations in these animal models must be acknowledged. First, the treatment period, 3 days for rabbits and 5 days for mice, does not equate to the timeline of when human patients have cerebral bleeding side effects. Human patients typically observe side effects after 1 year

[2–5]. I chose the dosing timeframe in this dissertation based on maximum platelet inhibition. Therefore, not all the adverse effects associated with clopidogrel may have been observed in these studies. Secondly, the rabbits (12-14 weeks) and mice (16-18 weeks) were relatively young and were not the most clinically representative model since clopidogrel is typically prescribed to patients with a mean age of 69 years old [33]. For rabbits and mice to be more comparable in age they would have needed to be closer to 9 years old and 2 years old, respectively. To further define the adverse effects of clopidogrel these studies should be repeated with aged mice closer to 2 years old. Another limitation to the animal models is the unavailability of a $P2Y_{12}^{-/-}$ rabbit. The effects we observed in rabbit MCAs may have not been observed in mouse PCAs due to species differences. $P2Y_2$ receptor expression has not been analyzed in mouse cerebral arteries. Previous reports have demonstrated that $P2Y_2$ is expressed in rabbit cerebral arteries [34]. Additionally, I have demonstrated, pharmacologically, that $P2Y_2$ is expressed in rabbit cerebral vessels. I was unsuccessful in elucidating if $P2Y_2$ is indeed expressed in mouse cerebral vessels.

The studies in Chapter 1 - Part 2 and Chapter 2 were done with male rabbits. Additionally, the experiments done in Chapter 3 were completed with male mice. There were no differences between sexes in any of my studies in Chapter 4, however, this must be further evaluated as there was not a large enough group number for complete analysis when separated by sex. Typically, men have a higher incidence of intracerebral hemorrhage and have an increased risk of expansion and mortality [35,36]. However, women have a reported higher incidence of bleeding on clopidogrel treatment compared to men, but there is no difference in platelet activity between the sexes [37–40]. Interestingly, $P2Y_{12}$ mRNA expression in the brain is not different between male and female mice at 16 weeks old [41]. However, $P2Y_2$ mRNA expression in the brain is increased in females [41]. To determine if the increased bleeding observed in females is due to increased expression of $P2Y_2$, sex differences in blood loss and $P2Y_2$ vessel function need to be assessed.

There were also limitations with the design of some experiments. Consistency between blood loss measurements is difficult. In rabbits, I used the best available model to produce reproducible results, which is through a uniform incision on the tongue with a Surgicutt® device [42,43]. However, whisking away the blood from the incision by the operator may compromise the assay. To compensate for this, the blood is blotted next to the incision to not disturb clot formation. In mice, the force of tail transection can be variable. Unfortunately, there is not a standardized device to make this amputation. To decrease variability, I used a new scalpel and transected the measured tail with a downward force. Additionally, for both rabbits and mice, I performed each bleeding time/blood loss measurement to remove any interpersonal variables. Pressure myography, while widely used to study the vascular structure and function *ex vivo*, diminishes the effects of blood viscosity, flow, and interactions of blood components with the vessel wall. The PCAs from mice did not have flow through, while the MCAs from rabbits did have flow through. This could cause the results to be different as previous studies demonstrate that flow induces relaxation [44,45]. Furthermore, we used UTP, a nonselective $P2Y_2$ and $P2Y_4$ agonist, which does not adequately elucidate specific $P2Y_2$ inhibition by clopidogrel.

4. Future Directions

Several studies need to be conducted to fully elucidate the pleiotropic effects of clopidogrel. The next step in the progression of these studies is to assess PCA function in $P2Y_{12}^{-/-}$ mice using a more selective $P2Y_2$ agonist. Unfortunately, the PCAs did not respond to the commercially available selective $P2Y_2$ agonist, MRS 2768. Therefore, in chapter 4 I decided to use the semi-selective agonist, UTP, which activates both $P2Y_2$ and $P2Y_4$. I propose that 10 µM NF157, a $P2Y_{11}$ antagonist, should be circulating in the bath, and then increasing concentrations of ATPγS (10^{-9}-10^{-5} M), a non-hydrolyzed ATP analog, will be added to observe $P2Y_2$ inhibition only [46]. Unfortunately, at the time of my study, NF157 was not available and therefore was not a method I could pursue. ATPγS, at these concentrations, activates both

$P2Y_2$ and $P2Y_{11}$. Therefore by adding NF157 first, we can inhibit any effects associated with $P2Y_{11}$ to observe the $P2Y_2$ specific effects. This study will determine if the $P2Y_2$ inhibition observed with clopidogrel treatment in rabbits also occurs in mice.

The studies described in Chapter 4 demonstrate that clopidogrel-treated wild-type mice and vehicle-treated $P2Y_{12}^{-/-}$ mice had an increase in blood loss compared to vehicle-treated wild-type mice. However, I was not able to replicate the finding, reported by André et al., that clopidogrel-treated $P2Y_{12}^{-/-}$ mice had increased blood loss compared to vehicle-treated $P2Y_{12}^{-/-}$ mice [8]. In my studies, clopidogrel treatment did not potentiate blood loss in the $P2Y_{12}^{-/-}$ mice. I performed tail blood loss in Drabkin's reagent to measure hemoglobin instead of measuring blood volume, therefore I followed the protocol described by Saito et al [47]. This protocol uses a 5 mm tail amputation which did not mimic the results described by André et al. with a 2 mm tail amputation. The extra 3 mm tail amputation I implemented could be overly robust, thereby resulting in the reduced sensitivity observed between my data and previous reports. Therefore, the next step in these studies is to optimize tail amputation to tease apart the differences observed with blood loss experiments. I propose reducing the tail amputation to 2 mm as well as the duration of bleeding to 15 minutes would allow for small changes in blood loss to be observed. I hypothesize my experiments conducted with 5 mm tail loss and 30-minute bleeding duration reached the maximum blood loss and therefore hid the differences in bleeding between vehicle- and clopidogrel-treated $P2Y_{12}^{-/-}$ mice. Optimizing this study would aid in elucidating the minor bleeding changes and validate if clopidogrel indeed potentiates bleeding in $P2Y_{12}^{-/-}$ mice.

The body regulates blood pressure by several different means. One of those mechanisms is through mechanoreceptors which are activated upon the stretch of the vessel [48]. The way a vessel adapts to increased flow rates determines blood pressure. In normal states, increased blood flow activates the endothelium to release vasodilatory mediators. This increase in vessel diameter maintains the blood pressure. Interestingly, activation of $P2Y_2$ is crucial for flow-induced relaxation [49]. Loss of endothelial $P2Y_2$ increases arterial blood pressure and

decreases phosphorylation of endothelial nitric oxide synthase [49], thereby indicating that endothelial $P2Y_2$ is important for the flow-induced generation of nitric oxide (NO), which is essential in blood pressure regulation. Clopidogrel's effects on blood pressure, flow-induced relaxation, and NO production should be examined. This would determine if the inhibition of $P2Y_2$ by clopidogrel has detrimental effects on blood pressure. These studies are critical as many of the patients taking clopidogrel are also hypertensive. To date, the effects clopidogrel has on blood pressure in patients have not been evaluated. I hypothesize clopidogrel, through the inhibition of $P2Y_2$, decreases NO production thereby increasing blood pressure.

Additionally, changes in collagen deposition within the cerebral vasculature need to be assessed. Collagen plays an important role in maintaining the elasticity and structure of the vessel by limiting distensibility [50]. An increase in collagen in the vessel wall leads to increased stiffness [51]. These studies would elucidate whether clopidogrel treatment and the knockdown of $P2Y_{12}$ increase collagen deposition, thereby causing decreased distensibility and increased arterial stiffness, or if the changes to the vascular structure are collagen-independent. If collagen deposition is unchanged, other mechanisms by which arteries become stiff will be investigated. Arterial wall stiffness is also determined by elastin cross-linking, endothelial dysfunction, and vascular smooth muscle cell stiffness [52]. The regulation of actin polymerization and focal adhesion have been associated with vascular stiffness [53]. Furthermore, an increase in vascular smooth muscle cell adhesion to the extracellular matrix increases arterial stiffness [54]. To elucidate the mechanism to which arterial wall stiffness is increased with clopidogrel treatment, actin polymerization, cell adhesion, and vascular cell death need to also be assessed.

The increased risk of intracerebral hemorrhage with clopidogrel treatment must be evaluated to determine the link between the vascular changes I discovered and bleeding. Commonly, intraparenchymal injection of bacterial collagenase is used to induce experimental intracerebral hemorrhage [55]. Collagenase enzymatically disrupts the basal lamina of brain capillaries to cause an active bleed. This model accurately replicates what is seen in humans

as most hemorrhagic strokes occur in the basal ganglia [27]. The use of this model with and without clopidogrel treatment would determine if clopidogrel increases intracerebral hemorrhage. However, the use of clopidogrel alone would be impossible to identify if increased intracerebral bleeding is due to the antiplatelet properties or via off-target effects. Therefore, it is imperative to also assess other antiplatelet therapeutics such as ticagrelor, prasugrel, and DT-678. If clopidogrel increased intracerebral hemorrhage, but the other antiplatelet therapies did not, it would provide further evidence that clopidogrel potentiates bleeding in a platelet-independent mechanism.

The mechanism to which endothelial $P2Y_2$ activation causes constriction is still unknown. However, it is known that uridine adenosine tetraphosphate (Up_4A) activates $P2Y_2$ to induce vasoconstriction [56,57]. When endothelial cells are subjected to sudden stretch, activated purinergic receptors release Up4A and other endothelium-derived contracting factors (EDCFs). These EDCFs diffuse to the VSMCs and initiate contraction [58]. The mechanism to which activation of endothelial $P2Y_2$, via Up4A, results in constriction needs to be assessed in future studies. If Up4A is not involved in the activation of endothelial $P2Y_2$ other EDCFs must be evaluated such as endothelin-1 or thromboxane.

While the future studies I have described thus far would further elucidate the off-target effects of clopidogrel, clopidogrel is prescribed to patients with a low dose of aspirin. To mimic the side effects observed clinically the experiments I have described in this dissertation should be repeated in the presence of aspirin. The results from these experiments would determine if the added inhibition of cyclooxygenase by aspirin contributes to the adverse bleeding associated with clopidogrel treatment.

5. Final Perspectives

Current antiplatelet therapeutics are efficacious in preventing arterial thrombosis but are accompanied by detrimental side effects. Understanding the mechanism associated with

clopidogrel bleeding is critical for the development of novel antiplatelet agents with reduced adverse effects. There is an immediate need to elucidate these clopidogrel off-target effects as more than 4 million patients are estimated to be on clopidogrel therapy in the United States alone [59]. I have outlined several adverse effects associated with clopidogrel use in both humans and animals, however, this dissertation focused on adverse bleeding. My studies led me down the path of determining how vessel function could be altered thereby contributing to bleeding. I proposed that clopidogrel would impair purinergic signaling in the vasculature. Additionally, I proposed that the adverse bleeding associated with clopidogrel was $P2Y_{12}$-independent. My studies show that 1) $P2Y_2$ is inhibited in MCAs in rabbits and that 2) $P2Y_2$ is not inhibited by the clopidogrel prodrug, the M1 metabolite, or the M2 metabolite. 3) I discovered the M1 metabolite, M2 metabolite, and M13 metabolite are not responsible for the adverse bleeding. 4) I also demonstrate that $P2Y_{12}^{-/-}$ mice and clopidogrel-treated mice have decreased distensibility and increased arterial stiffness. Overall, this work has provided new information as to how clopidogrel treatment causes adverse bleeding.

Even though I have just described all of the detrimental side effects associated with clopidogrel, it is still the safest option for antiplatelet therapy. The studies described in this dissertation will not discontinue the use of clopidogrel, however, it will help aid in the development of new antiplatelet agents. Importantly, the active metabolite of clopidogrel does not seem to be the cause of the adverse bleeding. A conjugate of the active metabolite of clopidogrel, such as DT-678, would be a safer next-generation antiplatelet therapy. Even if a novel antiplatelet agent is developed, my studies stress the importance of simplified metabolism with little to no additional metabolites produced to minimize off-target effects.

Acknowledgments

I would like to thank Adam Lauver and Nathan Tykocki for their proofreading and helpful suggestions.

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