Characterization of the widely used Rac1-inhibitors NSC23766 and EHT1864 in mouse platelets

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Untersuchung der Rac1-Inhibitoren NSC23766 und EHT1864 in murinen Thrombozyten

Doctoral thesis for a medical doctoral degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
Section: Biomedicine

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Würzburg, 2017

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Summary

Platelet activation and aggregation at sites of vascular injury is critical to prevent excessive blood loss, but may also lead to life-threatening ischemic diseases, such as myocardial infarction and stroke. Extracellular agonists induce platelet activation by stimulation of platelet membrane receptors. Signal transduction results in reorganization of the cytoskeleton, shape change, platelet adhesion and aggregation, cumulating in thrombus formation. Several Rho GTPases, including Rac1, Cdc42 and RhoA, are essential mediators of subsequent intracellular transduction of ITAM- and GPCR-signaling. Therefore, inhibition or knockout can result in severely defective platelet signaling.

Mice with platelet specific Rac1-deficiency are protected from arterial thrombosis. This benefit highlights further investigation of Rac1-specific functions and its potential as a new pharmacological target for prevention of cardiovascular diseases. Two newly developed synthetic compounds, NSC23766 and EHT1864, were proposed to provide highly specific inhibition of Rac1 activity, but both drugs have never been tested in Rac1-deficient cell systems to rule out potential Rac1-independent effects.

This study revealed significant off-target effects of NSC23766 and EHT1864 that occurred in a dose-dependent fashion in both wild-type and Rac1-deficient platelets. Both inhibitors individually affected resting platelets after treatment, either by altering membrane protein expression (NSC23766) or by a marked decrease of platelet viability (EHT1864). Platelet apoptosis could be confirmed by enhanced levels of phosphatidylserine exposure and decreased mitochondrial membrane potential. Phosphorylation studies of the major effector proteins of Rac1 revealed that NSC23766 and EHT1864 abolish PAK1/PAK2 activation independently of Rac1 in wild-type and knockout platelets, which may contribute to the observed off-target effects.

Additionally, this study demonstrated the involvement of Rac1 in G protein-coupled receptor-mediated platelet activation and GPIb-induced signaling. Furthermore, the data revealed that Rac1 is dispensable in the process of integrin αIIbβ3-mediated clot retraction.

This study unveiled that new pharmacological approaches in antithrombotic therapy with Rac1 as molecular target have to be designed carefully in order to obtain high specificity and minimize potential off-target effects.

Zusammenfassung

Die Aktivierung und Aggregation von Thrombozyten nach Gefäßverletzungen ist entscheidend um starken Blutverlust zu vermeiden. Allerdings können diese Prozesse auch zu lebensbedrohlichen ischämischen Erkrankungen führen, wie beispielsweise Myokardinfarkt und Schlaganfall. Die Stimulation der Membranrezeptoren durch Triggersubstanzen leitet die Thrombozytenaktivierung und somit die Reorganisation des Zytoskeletts ein. Dies ermöglicht die Adhäsion und Aggregation der Thrombozyten und führt letztendlich zur Thrombusbildung. Die Rho GTPasen Rac1, Cdc42 und RhoA sind als wichtige Mediatoren an der intrazellulären Signaltransduktion beteiligt. Eine medikamentöse Hemmung oder ein genetischer Knockout kann daher die intrazellulären Signalkaskaden so stark beeinträchtigen, dass eine effiziente Aktivierung der Thrombozyten nicht mehr möglich ist. In Mäusen mit thrombozytenspezifischem Knockout von Rac1 wurde festgestellt, dass der Funktionsverlust von Rac1 gleichzeitig auch Schutz vor der Entwicklung von arterieller Thrombose bedeutet. Könnte man sich diese Tatsache pharmakologisch zunutze machen, würde die Hemmung von Rac1 möglicherweise einen neuen, erfolgsversprechenden Ansatz in der Prävention von kardiovaskulären Erkrankungen darstellen.

Für den Forschungseinsatz wurden die zwei synthetischen Inhibitoren NSC23766 und EHT1864 entwickelt um Rac1-vermittelte Funktionen zu studieren. Beide Substanzen versprechen eine hochspezifische Hemmung der Rac(1)-Aktivität, wurden bisher jedoch nicht in Zellsystemen mit Rac1-Defizienz verwendet um die Substanzen kritisch auf mögliche, unerwünschte Nebenwirkungen zu untersuchen.

In dieser Dissertation wurde gezeigt, dass NSC23766 und EHT1864 zwar effektive Hemmstoffe für Rac1 sind, allerdings genauso Rac1-unabhängige Nebenwirkungen verursachen. Beide Hemmstoffe führten zu Veränderungen der Thrombozyten: Während unter NSC23766 eine verminderte Expression von Membranrezeptoren beobachtet wurde, führte EHT1864 zu einer stark beeinträchtigten Vitalität der Thrombozyten. Anhand von erhöhten Phosphatidylserin-Werten und einer Veränderung des mitochondrialen Membranpotenzials in den behandelten Thrombozyten konnte die EHT1864-vermittelte Apoptose nachgewiesen werden. Letztendlich wurde anhand der verminderten Phosphorylierung von PAK1/PAK2

gezeigt, dass die Aktivierung dieser Rac1-Effektorproteine durch NSC23766 und EHT1864 direkt unterdrückt wird.

Zusätzlich zu den Inhibitor-vermittelten Effekten wurde anhand von Rac1-defizienten Thrombozyten nachgewiesen, dass Rac1 auch an GPCR- und GPIb-vermittelten Signalkaskaden beteiligt ist. Außerdem wurde beobachtet, dass Rac1 für die Integrin αIIbβ3-vermittelte *clot retraction* entbehrlich ist.

Die Ergebnisse dieser Studie legen dar, dass neue pharmakologische Substanzen für die antithrombotische Therapie mit Rac1 als Zielmolekül gründlich erforscht und hinterfragt werden müssen um die Spezifität zu maximieren und vor allem das Nebenwirkungsprofil zu minimieren.

1 Introduction

Platelets are anucleate discoid-shaped cell fragments with a diameter of 3 – 4 µm in humans and 1 – 2 µm in mice. Platelets are continuously produced in the bone marrow by their nucleated precursor cells, the *megakaryocytes* (MK), and released into the blood stream. Platelets are the second most abundant circulating blood cells (150 – 400 x^3 platelets/µl in humans and up to 1000 x 10^3 platelets/µl in mice), and the majority of them never undergo activation and adhesion before they are removed from the circulation. Murine platelets have a limited average lifespan of 5 days (10 days in humans). Aged, pre-activated and dysfunctional platelets are removed from the circulating blood by macrophages in the liver and spleen[1]. Despite being anuclear, platelets are metabolically active cells, containing functional cellular organelles and mRNAs and thus, platelets are able to translate and renew certain proteins to a limited extent. The platelet cytoplasm contains different types of secretory granules, among others most notably α- and δ-granules. The most abundant platelet granules are α-granules, which store a variety of adhesion proteins, such as fibrinogen, growth factors, *von Willebrand Factor* (vWF), coagulation factors and chemokines. Dense granules store a plethora of small inorganic molecules which act as second wave mediators during platelet activation, including *adenosine di- and triphosphate* (ADP, ATP), Ca^{2+}, histamine and serotonin[2].

Platelets are the key regulators of primary hemostasis at sites of endothelial injury after exposure of *extracellular matrix* (ECM) components. Exposed ECM-components induce platelet activation with subsequent adhesion and formation of platelet aggregates. A platelet plug is formed in order to seal vessel lesions and to minimize further blood loss. However, platelet activation is not only triggered by traumatic damage of vessels. Many widespread diseases like obesity, diabetes and diseases of the cardiovascular system cause vessel wall inflammation and may trigger pathological platelet activation. In this case thrombus formation can lead to acute or chronic vessel occlusion in multiple organs, frequently inducing myocardial infarction and ischemic stroke, which currently represent the leading causes of death and severe morbidity worldwide[3,4]. In order to limit thrombus formation under these pathological conditions, inhibitors which affect platelet degranulation and the release of adhesion receptors have always been a major interest of research. Today

antiplatelet agents like the *cyclooxygenase* (COX)-inhibitor *acetylsalicylic acid* (ASA) and the ADP-receptor inhibitor clopidogrel and their derivatives are widely used drugs that successfully prevent thrombus formation; however, with the important caveat – the increased bleeding risk as a major side effect during the treatment of human patients[5]. Therefore, it is of great importance to find new approaches to prevent pathologic platelet activation and thrombus formation without increased bleeding risk.

1.1 Platelet activation and thrombus formation

Upon vessel injury, multiple processes are induced in order to stop further bleeding and minimize blood loss. Activation of primary hemostasis initiates platelet recruitment, adhesion and thrombus formation and can be subdivided into three major steps (Figure 1-1).

(1) In the first step, circulating platelets are decelerated by the initial contact to the exposed ECM, known as tethering. This capture is mediated by interaction of the platelet *glycoprotein* (GP) Ib-V-IX receptor complex with vWF, which is immobilized on the collagen surface of the ECM[6]. Although this GPIbα-vWF interaction is essential for the initial capture, it cannot induce firm platelet adhesion onto the exposed ECM. It rather leads to a rapid deceleration, which results in platelets 'rolling' on the ECM. (2) In a second step, deceleration of the platelets allows interaction of GPVI, the major platelet collagen receptor, with collagen and leads to a stable platelet adhesion on the ECM[7]. GPVI-ligation initiates an intracellular signaling cascade that triggers activation of platelet integrins and induces cytoskeletal rearrangement, the mobilization of α- and δ-granules and the subsequent release of secondary messengers, particularly ADP and *thromboxane A$_2$* (TxA$_2$)[8,9].

Together with thrombin, these mediators induce further platelet activation by binding to *G protein-coupled receptors* (GPCRs) in an autocrine and paracrine manner and recruit further platelets from the circulating blood to the growing thrombus[10]. Finally, these pathways lead to the conformational change of integrin adhesion receptors, especially the abundantly expressed integrin αIIbβ3. The conformational change of the integrins induces a shift from low to a high affinity state. (3) In the third step, integrin αIIbβ3 also binds to fibronectin and vWF and thereby reinforces a firm adhesion of the platelets to the ECM. Furthermore binding of integrin αIIbβ3 to

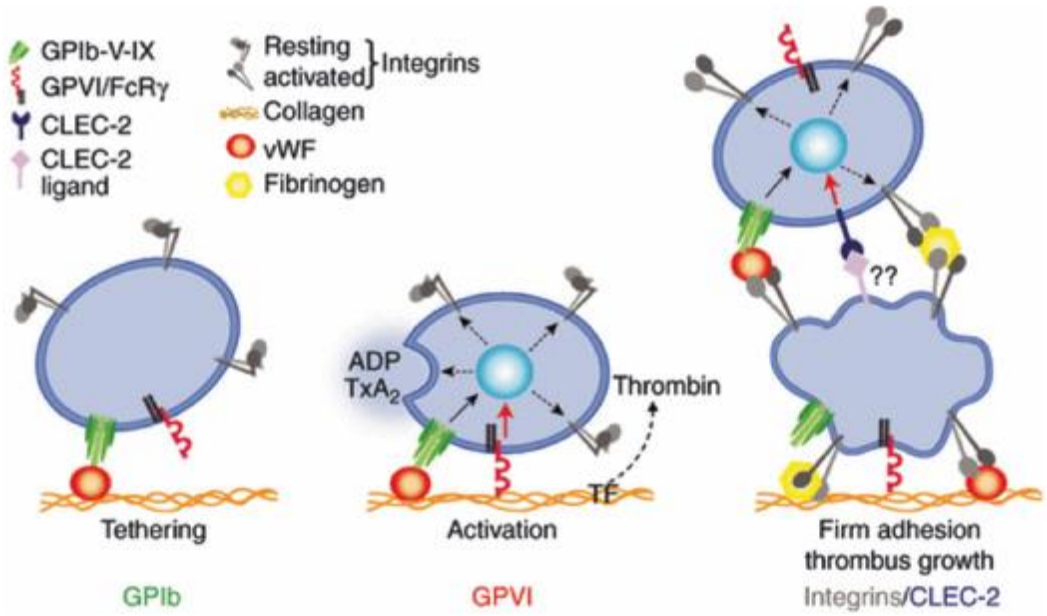

Figure 1-1: Simplified model of platelet tethering, adhesion and thrombus formation on ECM. During endothelial damage ECM proteins get exposed to the blood flow. vWF is immobilized on exposed collagen and interacts with the GPIb-V-IX receptor complex of circulating platelets (tethering). This leads to rapid platelet deceleration and allows interaction of platelet GPVI with the exposed collagen. GPVI signaling initiates platelet activation and conversion of integrins to a high-affinity state. Secondary messengers such as ADP and TxA_2 are released and lead to platelet activation in autocrine and paracrine manner. Exposed TF induces thrombin generation, which in turn reinforces platelet activation via GPCRs. Firm adhesion is achieved by activation of integrins, which bind fibrinogen and vWF at the ECM and furthermore by bridging between adjacent platelets. ADP, *adenosine diphosphate*; TxA_2, *thromboxane* A_2; vWF, *von Willebrand Factor*; TF, *tissue factor*; GPCR, *G protein-coupled receptors*; CLEC-2, *C-type lectin-like receptor type 2*. Taken from: Nieswandt et al., *J Thromb Haemost*, 2011[63].

fibrinogen and vWF mediates bridging of adjacent platelets and contributes to thrombus stabilization[8,11,12]. Additionally, ligand-occupied integrin αIIbβ3 mediates outside-in activation of platelets, in which multiple signaling pathways result in cytoskeletal reorganization, platelet spreading and clot retraction[12,13].

1.2 Signaling events during platelet activation

Platelet signaling can be induced by synergistic activation of several membrane receptors, which accelerate enzyme activity and/or modify subcellular localization of *phospholipase C* (PLC) isoforms (Figure 1-2). The active PLC-isoforms initiate hydrolysis of *phosphatidylinositol-4,5-bisphosphate* (PIP_2) to *inositol-3,4,5-triphosphate* (IP_3) and *diacylglycerol* (DAG) which in turn elevates the cytosolic Ca^{2+} concentration and triggers subsequent integrin activation, shape change, aggregation and secretion. Two major signaling pathways can be distinguished: Activation via

GPCRs and activation via *immunoreceptor tyrosine-based activation motif* (ITAM)-bearing receptors.

Platelet activation via GPCRs is conducted by several G protein-mediated signaling pathways which are mainly initiated by three types of heterotrimeric G proteins

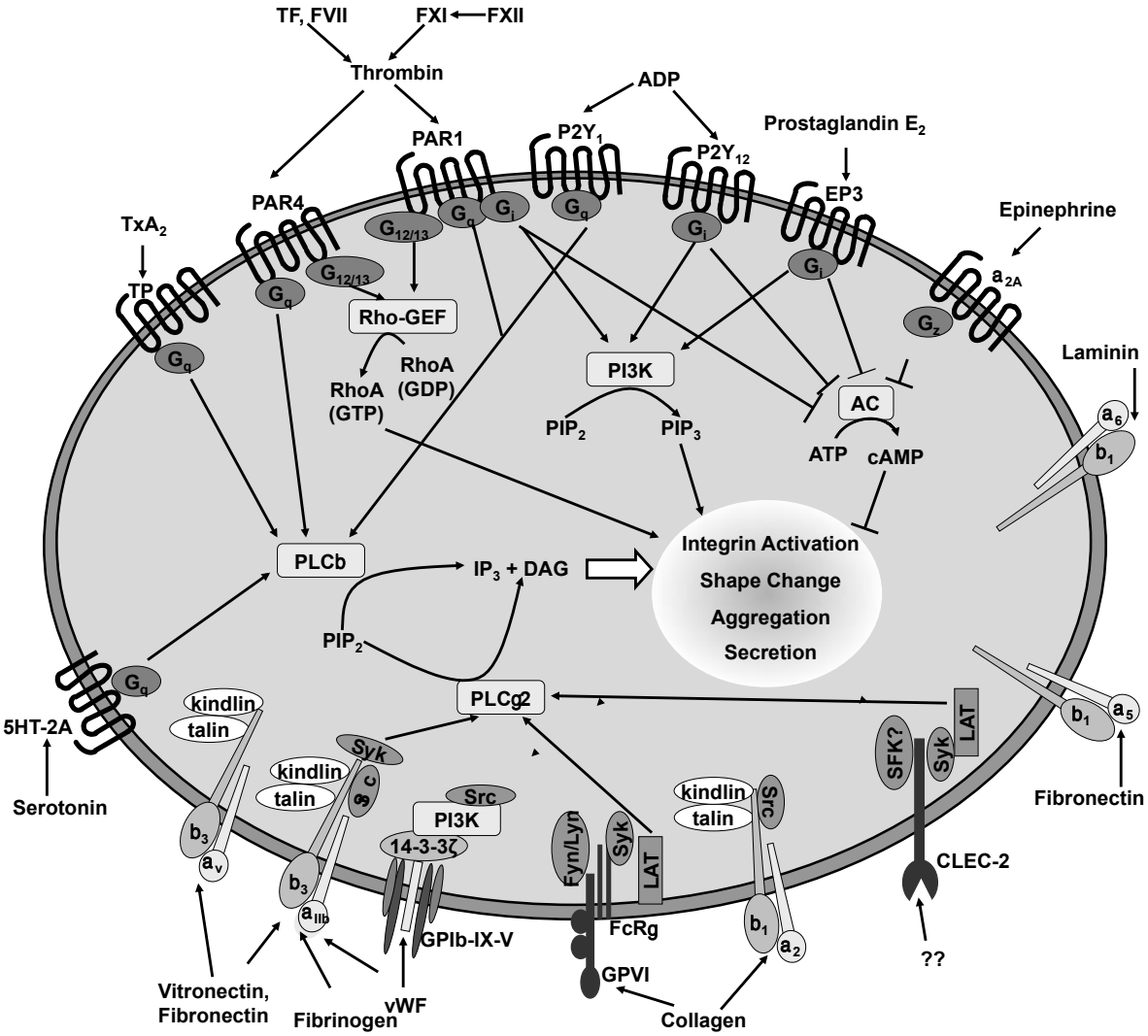

Figure 1-2: Major signaling pathways in platelets. Second wave mediators, including ADP, TxA_2 and thrombin serve as agonists for heterotrimeric G protein-coupled receptors (G_q, $G_{12/13}$, G_i) and activate downstream effectors. Stimulation of integrins as well as the (hem)ITAM receptors GPVI and CLEC-2 induces PLCγ2 activation. Both signaling pathways culminate in elevation of intracellular Ca^{2+}, which in turn triggers integrin activation, platelet shape change, aggregation and secretion. ADP, *adenosine diphosphate*; GDP, *guanosine diphosphate*; GTP, *guanosine triphosphate*; GEF, *guanine nucleotide exchange factor*; PAR, *protease-activated receptor*; CLEC-2, *C-type lectin like receptor 2*; Fg, *fibrinogen*; TF, *tissue factor*; TxA_2, *thromboxane A2*; vWF, *von Willebrand Factor*; RhoA, *Ras homolog gene family, member A*; LAT, *linker for activation of T cells*; Syk, *spleen tyrosine kinase*; SFK, *Src family kinase*; PLC, *phospholipase C*; PI3K, *phosphatidylinositol-3-kinase*; PIP_2, *phosphatidylinositol-4,5-bisphosphate*; PIP_3, *phosphatidylinositol-3,4,5-trisphosphate*; IP3, *inositol-3,4,5-trisphosphate*; AC, *adenylyl cyclase*; DAG, *diacylglycerol* (taken from: Stegner, Nieswandt, *J Mol Med*, 2011[90]).

downstream of the platelet receptor: (1) G_q induces signaling and phosphorylation of PLCβ[10]. (2) $G_{12/13}$ transduces signaling through RhoGTPases. (3) G_i leads to activation of *phosphoinositol-3-kinase* (PI3K). GPCR signaling is triggered by soluble agonists like ADP and TxA_2, which are released during thrombus formation, as well as thrombin. ADP binds to the GPCRs $P2Y_1$ and $P2Y_{12}$, which are coupled to G_q and G_i subunits. The TxA_2 receptors (TP) and the thrombin binding *protease-activated receptors* (PARs) are mainly coupled to G_q and $G_{12/13}$ subunits. In human platelets PAR1 and 4 isoforms are expressed, whereas mouse platelets exhibit a different expression profile of PARs (3/4)[14,15]. Platelet stimulation with thrombin, TxA_2 or ADP induces secretion of secondary messengers, which amplify platelet response by a positive feedback.

Notably, the $P2Y_{12}$ receptor serves as target structure for the potent antiplatelet agents clopidogrel, prasugrel, ticagrelor and their derivatives. In clinical practice these antiplatelet agents are used to prevent thrombus formation. Together with the thromboxane synthesis inhibitor ASA, $P2Y_{12}$ receptor antagonists are used, in line with therapeutic guidelines, as secondary preventive therapy after myocardial infarction and stroke.

The other major signaling pathway is initiated by the platelet receptor GPVI, an *immunoreceptor tyrosine-based activation motif* (ITAM)-bearing receptor. Stimulation with collagen powerfully activates platelets via GPVI binding, inducing a downstream signaling cascade that culminates in the activation of PLCγ2[16].

The ITAM is a highly conserved amino acid sequence, encoding two tyrosine phosphorylation sites. The tyrosine residues become activated by phosphorylation upon ligand occupation of the (hem)ITAM-receptor. Thereby an intracellular downstream signaling cascade is initiated. Murine platelets express two types of ITAM-containing receptors: the Fc receptor γ chain (FcR γ) which functionally associates with the GPVI receptor, and the hemITAM receptor *C-type lectin-like receptor 2* (CLEC-2)[17], whereas human platelets express a third ITAM, the FcγRIIA, which shares common downstream receptors with GPVI and CLEC-2[18].

GPVI is the central activating receptor during collagen-mediated thrombus formation. GPVI is a 62 kDa MK-/platelet-specific transmembrane protein that belongs to the superfamily of *immunoglobulin* (Ig) surface receptors[19]. GPVI is non-covalently associated with a homodimeric FcR γ-chain, and each chain contains one copy of a

classical ITAM, which serves as the signaling subunit of the receptor complex. Ligand occupation of GPVI leads to crosslinking and subsequent phosphorylation of the two tyrosine residues in the ITAM on the FcR γ-chain by the *Src family tyrosine kinases* (SFK) Lyn and Fyn[20]. After tyrosine phosphorylation of the ITAM signaling is transduced by phosphorylation and activation of the *spleen tyrosine kinase* (Syk) and the *linker for activation of T-cells* (LAT), culminating in the phosphorylation of the effector proteins PLCγ2 and *phosphoinositol* (PI)-3-kinase[21].

Activation of either (hem)ITAM or GPCR signaling pathways results in the activation of PLC isoforms, which hydrolyze the membrane phospholipid PIP_2 to IP_3 and DAG. DAG triggers Ca^{2+} influx via activation of *protein kinase C* (PKC) and the *transient receptor potential channel* (TRPC)[22]. IP_3 binds to Ca^{2+} channels of the intracellular Ca^{2+} stores, resulting in *store-operated Ca^{2+} entry* (SOCE). Both pathways trigger marked elevation of intracellular Ca^{2+} levels, which is the central step for platelet activation, granule secretion, shape change and aggregation[23].

1.3 Small GTPases of the Rho family

Small GTPases of the Rho family belong to the Ras-related superfamily and are *guanosine-5'-triphosphate* (GTP) binding proteins that act as molecular switches by cycling between an inactive *guanosine diphosphate* (GDP)-bound and an active GTP-bound state (Figure 1-3). Small GTPases have an intrinsic GTPase activity that leads to hydrolysis of GTP to GDP and allows the temporal on-off switch of their effector proteins[24]. Rho GTPases are key regulators of cytoskeletal rearrangement, thereby modulating platelet activation, aggregation response, granule secretion, spreading and thrombus formation[25,26]. In the inactive state, the Rho GTPases are bound to GDP with high affinity and a slow dissociation rate. Several isoforms of *Guanine nucleotide-exchange factors* (GEFs) regulate the exchange of GDP to GTP by facilitating the dissociation of GDP. After GEF binding to Rho GTPases, the GDP nucleotide is released and subsequently replaced by GTP, thereby activating the Rho GTPase[27]. The duration of activity is limited by *GTPase-activating proteins* (GAPs), which accelerate the hydrolysis of GTP to GDP and switch the Rho GTPase into inactive state[27]. A third set of regulatory proteins are the *Guanine nucleotide-dissociation inhibitors* (GDIs), which bind to Rho GTPases with their hydrophobic pocket, keeping the GTPase away from effector proteins in order to prevent spontaneous activation[28,29]. Active GTP-bound Rho GTPases interact with effector

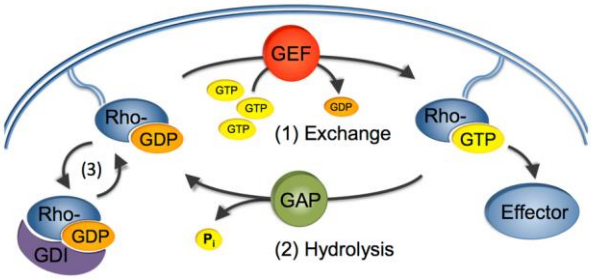

Figure 1-3: The Rho GTPase cycle. Rho GTPases cycle between an inactive, GDP-bound, and an active, GTP-bound state in which they can interact with effector molecules. GEFs mediate the exchange (1) of GDP to GTP. GAPs trigger the hydrolysis (2) of Rho-GTP to RhoGDP and thus inhibit Rho GTPase activation. GDIs form soluble complexes with Rho-GDP and prevent spontaneous activation (3). GEFs, *guanine nucleotide-exchange factors*; GAPs, *GTPase-activating proteins;* GDIs, *guanine nucleotide-dissociation inhibitors* (modified from Aslan *et al*., *J Thromb Haemost*, 2013[24]).

proteins serving as temporal on-off switches and thereby transduce downstream receptor signaling. Rho GTPases represent essential modulator proteins for cell adhesion, microtubule dynamics and vesicle transport[26,29]. Since platelets contain limited numbers of RhoGTPases, GEF, GDI and GAP isoforms, they serve as an important system for investigation of Rho GTPase function.

Rho GTPases have been shown to play a central role in platelet signaling and thus they have been in focus of platelet research for a long time. Most of the knowledge about the role and function of the various Rho GTPases has been gained by overexpression studies, knockout models or pharmacological inhibition. Studies have been performed in platelets and other human and murine cell systems. It has to be taken into account that human platelets have a different protein expression profile than murine platelets and not all generated data are easily transferrable between different cell systems[15]. In recent years several mouse models with tissue- or lineage-specific deletion of Rho GTPases have been developed, greatly facilitating the investigation of these proteins.

The family of Rho GTPases comprises over 20 members. Among them, Rac1 (*Ras-related C3 botulinum toxin substrate 1*), Cdc42 (*cell division control protein 42*) and RhoA (*Ras homolog gene family, member A*) are the most studied GTPases in platelet biology. Activation of Rac1 and Cdc42 is associated with the control of lamellipodia and filopodia formation[30–33], respectively, whereas RhoA has been shown to be essential for stress fiber formation and platelet shape change[34].

1.3.1 Rac1

The Rac subfamily consists of the three isoforms Rac1, Rac2, Rac3 and the tumor-associated constitutively active isoform Rac1b[35,36]. Rac1 is expressed ubiquitously, while the Rac2 isoform can be found mainly in the hematopoietic cell system and Rac3 in the central nervous system.

In platelets Rac1 activity is mainly regulated by the specific GEFs Trio and Tiam1. Another GEF, Vav1, predominantly activates Rac isoforms but can also activate RhoA and perhaps Cdc42[37]. Trio and Tiam1 can bind to a specific groove, which has been identified as the key area of GEF-mediated Rac1 activation. A Trp56 residue inside this binding groove serves as the critical determinant of GEF discrimination, thereby assuring that Rac1 is activated by Trio and Tiam1, but not by other GEFs like PDZ-Rho (RhoA GEF), Lbc (Rho GEF) or Intersectin (Cdc42 GEF)[38].

Rac proteins have pivotal functions in the cardiovascular system. Rac1 not only plays an essential role in platelet physiology, but it has been reported that Rac1 activity is related to the development of hypertrophic heart failure and coronary heart disease [39,40]. In mouse platelets Rac1 is the only isoform expressed in a detectable amount on protein level. Rac2 can be found on mRNA level in megakaryocytes and platelets, but it was not detectable in Western blot analysis[30]. It is well established that Rac1 is a major regulator of the signal transduction downstream of the ITAM-coupled receptor GPVI[33].

Two different knockout strategies of Rac1 in mouse platelets have been published before. Crossing of mice in which the Rac1 gene is flanked by *loxP* sites (*Rac1$^{fl/fl}$*) with transgenic mice, which carry the *Mx-cre* transgene results in mice lacking Rac1 in MKs and platelets. Deletion of the Rac1 gene can be induced in these mice by injection of *polyinosinic-polycytidylic acid* (Poly I:C) at the age of 5-6 weeks[33]. Another strategy of conditional knockout is the crossing of *Rac1$^{fl/fl}$* mice with mice expressing a Cre enzyme under the control of the MK-/*platelet-specific factor* (PF)4 promotor, which leads to abolished protein expression in MKs and platelets[41].

In *Mx-cre* knockout platelets Pleines *et al.* could show that Rac1 is essential for PLCγ2 activation. Importantly, this effect was independent of PLCγ2 tyrosine phosphorylation. Interestingly, Rac1 function was dispensable for sufficient activation of PLCβ, which is a major effector protein downstream of GPCR signaling. Rac1 deficiency leads to defective Ca^{2+} mobilization initiated by GPVI-LAT-PLCγ2

signaling resulting in severely defective platelet adhesion and thrombus formation on collagen matrix *in vitro* and *in vivo*, respectively. Furthermore, Rac1 has been established as the critical Rho GTPase isoform for lamellipodia formation in platelets[30,33]. Notably, several studies reported opposing results[30,33,42]: By use of Mx-Cre-mediated gene deletion Pleines *et al*.[33] and McCarty *et al.*[30] have shown that Rac1 deficiency does not affect transduction of GPCR signaling, whereas Akbar *et al.*[42] stated a generally diminished granule secretion in platelets with Mx-Cre-mediated Rac1-deficiency under treatment with the Rac1-specific small molecule inhibitor NSC23766.

In human platelets it has been shown recently that Rac1 activity is also mediated by calcineurin, triggered by Ca^{2+} elevation downstream of GPCRs, thereby activating its effector protein *p21 activated kinase* (PAK)II, a major regulator of granule secretion[43].

It is important to note that platelet-specific Rac1-deficient mice using the PF4-cre system are protected from arterial thrombosis[33]. Thus, inhibition of Rac1 activity might represent a suitable approach for antithrombotic therapy. Several small-molecule inhibitors of Rac1 are commercially available for platelet research. However, only little is known about their specificity and efficacy in platelets and possible (off-target) effects on thrombus formation.

1.3.2 Cdc42 and RhoA

Upon platelet activation, RhoA mediates stress fiber formation and platelet shape change from discoid to spherical form[34,44]. *Rho-associated protein kinase* (ROCK) is one of the major effector proteins in RhoA signaling, inducing actin remodeling. Studies in MK/platelet-specific RhoA-deficient mice revealed that RhoA is essential for G_{13}-mediated shape change and contributes to G_{13}/G_q-dependent integrin αIIbβ3 activation, granule release and clot retraction[34].

Cdc42 is known to be a major regulator of filopodia formation, exocytosis and secretion. Recent data suggested that filopodia formation can be maintained independently of Cdc42 function by action of the newly characterized Rho GTPase Rif (RhoF)[45,46]. However, Rif plays a dispensable role for filopodia formation in platelets indicating that other Rho GTPase could fully compensate the lack of Rif function in this process[47]. In line with this, mouse platelets isolated from MK/platelet-specific Cdc42 knockout mice displayed decreased filopodia formation on vWF-coated surfaces, indicating a unique role for GPIb mediated signaling. Yet, Cdc42-

deficient platelets retain the ability to spread on surfaces covered with *collagen-related peptide* (CRP) coated surface, suggesting that signaling downstream of GPVI may not depend on Cdc42 and other Rho GTPase isoforms are involved in this process[24]. Furthermore, Cdc42 depletion led to an increased granule secretion along with enhanced aggregation *in vivo*[32].

In spite of the phenotypic differences in platelets, both Cdc42 knockout models have revealed that Cdc42 depletion leads to severely reduced platelet counts, demonstrating a crucial role of this isoform in thrombopoiesis.

1.4 Small molecule inhibitors of Rac1

So far, three different classes of selective Rac1 inhibitors have been developed affecting Rac1 activation in different ways: (1) inhibition of Rac1-GEF interaction, (2) inhibition of Rac1-effector interaction and (3) antagonistic effect on the NADPH oxidase activity regulated by Rac1[48]. To date, only inhibitors from group (1) and (2) have been used in platelet research.

1.4.1 Inhibition of Rac1-GEF interaction

The most widely used inhibitors of Rac1 are the compounds NSC23766 and EHT1864, which have been used in a variety of different cellular systems under *in vitro* and *in vivo* conditions.

NSC23766 has been described for the first time by Gao *et al.* in 2004[38]. Using a structure-based virtual screening approach, NSC23766 has been identified as the first chemical compound that specifically inhibits Rac1 activity. The chemical structure of NSC23766 fits into a surface groove on Rac1, which forms the key area of GEF-recognition. It centers a Trp56 residue which is responsible for the discrimination of the respective GEF[49]. Indeed, NSC23766 inhibits Rac1-binding to Trio or Tiam1 and prevents nucleotide exchange; however it does not affect Rac1-Vav1 interaction[38]. Rac1 is accumulating in an inactive GDP-bound state upon platelet activation and is thus unable to interact with its effector proteins (Figure 1-4A). In GST pull-down assays a 50 %-inhibition of Rac1-Trio interaction was achieved at concentrations of 50 µM (IC_{50} value)[38]. Nevertheless, in recent literature NSC23766 has been used in a wide range from 5 to 200 µM.

Based on the lead structure of NSC23766, a novel derivative EHop-016 was identified in 2012[50]. EHop-016 showed a 100-fold higher effectiveness than

NSC23766, selectively binding Rac1 and Rac3 at 2 - 4 µm. However Cdc42-activity was also affected at concentrations from 5 µM upwards[50]. To date, EHop-016 has not been tested in platelet-based assays.

1.4.2 Inhibition of Rac1-effector interaction

In 2005 Désiré *et al.*[51] published the compound EHT1864, which in contrast to NSC23766, targets all isoforms of the Rac subfamily. Binding of EHT1864 on Rac1 isoforms leads to a disruption of the GDP- and GTP-nucleotide binding and inactivates Rac1 (Figure 1-4B). Unlike upon treatment with NSC23766, interaction of Rac1 with specific GEFs and GDIs is not affected by EHT1864. EHT1864 inhibits the complex formation of Rac1 with its effector proteins, thereby blocking signal transduction. Shutes *et al*. discussed several molecular mechanisms of EHT1864: Interaction of Rac1 with this inhibitor may lead to a dissociation of GTP nucleotides from Rac1, thereby significantly affecting the Rac1-GDP:Rac1-GTP equilibrium in the cell. However, they also showed that the binding site of EHT1864 involves two loci called switch I and switch II, which as well are involved directly in Rac1 effector binding. Because of this, it was discussed that EHT may prevent interaction of Rac1 with its effectors directly, by occupation of these loci[52].

The potency of EHT1864 has been investigated in a cell culture model and was described at concentrations of 5 µM. However, to date it has been used in platelets

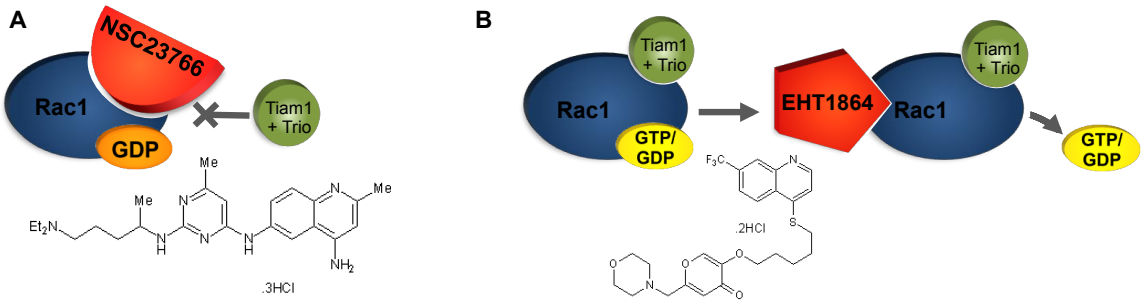

Figure 1-4: Mechanisms of Rac1-inhibition. (A) Competitive inhibition of Rac1-GEF interaction. Binding of NSC23766 to the surface groove inhibits interaction of Rac1 with its specific GEFs Tiam1 and Trio and prevents promotion of inactive Rac1-GDP to active Rac1-GTP. **(B)** Allosteric inhibition of nucleotide binding where Rac1 is left in inactive state. The detailed mechanism remains unclear. Binding of EHT1864 leads to disruption of the nucleotides GDP and GTP without interference of Rac1-GEF interaction. Another hypothesis is that EHT1864 interferes with the binding site of Rac1-specific effector proteins. Figures of molecular structures of NSC23766 and EHT1864 are from the respective Tocris Product Datasheets (Catalog No. 2161 and 3872).

only once by Stefanini *et al*., who investigated a potential crosstalk between Rap1 and Rac1 in human platelet activation at concentrations of 150 µM[43].

1.5 Aim of the study

The chemical compounds NSC23766 and EHT1864 have been used as Rac1 inhibitors in a variety of experimental settings. Pharmacological inhibition of Rac1 has emerged as a potential novel therapeutic approach in cardiovascular diseases. However, off-target effects of these drugs have not been investigated in Rac1-deficient cells. Depending on the used cell types and the experimental conditions, these drugs have been applied in a wide range of concentrations (10 - 300 µM) suggesting a suboptimal usage of the drugs by researchers. It has been shown that NSC23766 acts as a competitive antagonist of muscarinic acetylcholine receptors[53], thus questioning its potential use as a specific therapeutic agent. Therefore, it is essential to titrate the drug concentration in Rac1-deficient platelets and compare the outcome with wild-type platelets.

In this thesis I aimed to investigate the specificity and efficacy of NSC23766 and EHT1864 under *in vitro* conditions, using murine platelets isolated from wild-type and MK/platelet-specific Rac1-deficient mice.

2 Materials & Methods

2.1 Materials

2.1.1 Kits and Reagents

Reagent	Company
Adenosine diphosphate (ADP)	Sigma-Aldrich (Schnelldorf, Germany)
Apyrase type III	Sigma-Aldrich (Schnelldorf, Germany)
Botrocetin	Pentapharm Ltd. (Basel, Switzerland)
Bovine serum albumin (BSA)	AppliChem (Darmstadt, Germany)
β-mercaptoethanol	Roth (Karlsruhe, Germany)
Calcium chloride	Roth (Karlsruhe, Germany)
Collagen	Kollagenreagent Horm; Nycomed (Munich, Germany)
Convulxin	Enzo Life Sciences (Lörrach, Germany)
$DiOC_6$	Enzo Life Sciences (Lörrach, Germany)
EDTA	AppliChem (Darmstadt, Germany)
Enhanced chemoluminiscence (ECL) detection substrate	MoBiTec (Göttingen, Germany)
Epinephrine	Sigma (Deisenhofen, Germany)
EHT1864	Tocris Bioscience (Bristol, UK)
Dry milk, fat free	AppliChem (Darmstadt, Germany)
Fibrinogen from human plasma	Sigma-Aldrich (Schnelldorf, Germany)
Heparin sodium	Ratiopharm (Ulm, Germany)
HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid)	Life Technologies (Darmstadt, Germany)
High molecular weight heparin	Ratiopharm (Ulm, Germany)
Immobilon-P transfer membrane, PVDF, 0.45 µm	Merck Millipore (Darmstadt, Germany)
Indomethacin	Sigma-Aldrich (Schnelldorf, Germany)
Integrilin	Millennium Pharmaceuticals (Cambridge, MA, USA)
Isofluran CP®	cp-pharma (Burgdorf, Germany)
Magnesium chloride	Roth (Karlsruhe, Germany)
NSC23766	Tocris Bioscience (Bristol, UK)
Paraformaldehyde (PFA)	Roth (Karlsruhe, Germany)
Prostacyclin (PGI_2)	Calbiochem (Bad Soden, Germany)
Protease inhibitor cocktail	Sigma-Aldrich (Schnelldorf, Germany)
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich (Schnelldorf,

	Germany)
Sodium citrate	AppliChem (Darmstadt, Germany)
Thrombin	Roche Diagnostics (Mannheim, Germany)
U-46619	Alexis Biochemicals (San Diego, USA)

Collagen-related peptide was kindly provided by Dr. Steve Watson (University of Birmingham, UK). Rhodocytin was a generous gift from Dr. Johannes Eble (University Hospital, Frankfurt, Germany). All other non-listed chemicals were obtained from AppliChem (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma-Aldrich (Schnelldorf, Germany).

2.1.2 Antibodies

2.1.2.1 Purchased primary and secondary antibodies

Antibody	Company
goat anti-rabbit IgG–horseradish peroxidase	Cell Signaling, Danvers, MA, USA
p21-activated kinase (PAK)1	Cell Signaling, Danvers, MA, USA
PAK2	Cell Signaling, Danvers, MA, USA
PAK3	Cell Signaling, Danvers, MA, USA
phospho-PAK1	Cell Signaling, Danvers, MA, USA
phospho-PAK2 (Thr423/402)	Cell Signaling, Danvers, MA, USA
Rabbit anti-total-PLCγ2 (Q-20) (# sc-407)	Santa Cruz (Heidelberg, Germany)
Rabbit anti-phospho-PLCγ2 (Y759) (# 3874)	Cell Signaling (Danvers, MA; USA)

2.1.2.2 Monoclonal rat antibodies generated or modified in our laboratory

Antigen	Clone	Reference
α-GPVI	JAQ-1	[54]
α-α2	12C6	[54]
α-β1	Comm.	
α-CD9	97H11	unpublished
α-GPIb	15E2	[55]
α-GPIX	56F8	[56]
α-αIIbβ3 (GPIIb/IIIa)	14A3	[56]
α-GPV	89H11	[56]
CLEC-2	11E9	[57]

2.1.3 Mice

To generate mice lacking Rac1 in megakaryocytes (MKs)/platelets, mice containing the Rac1 gene flanked by loxP sites ($Rac1^{fl}$) were crossed with mice carrying the PF4-Cre transgene. $Rac1^{fl/fl\ PF4\text{-}Cre+}$ mice were used for experiments, and littermates ($Rac1^{fl/fl}$) served as controls. $Rac1/Cdc42^{-/-}$ mice were described previously, and $Rac1/RhoA^{-/-}$ mice were generated accordingly[58]. Efficient deletion of the respective proteins was shown by Western blot analysis (Figure 2-1). Mice were maintained on a mixed SV/129/C57/Bl-6 background. If not stated otherwise, 8-to-12-week-old mice of either sex were used in experiments. Animal studies were approved by the district government of Lower Franconia (Bezirksregierung Unterfranken).

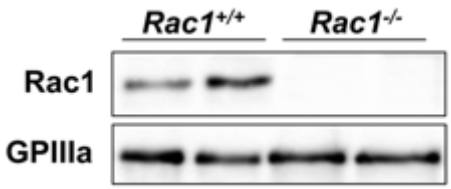

Figure 2-1: Analysis of Rac1 expression by Western blot. Blotting shows sufficient PF4-Cre mediated deletion of Rac1 (referred to as *Rac1*$^{-/-}$) as compared with wild-type platelets (referred to as *Rac1*$^{+/+}$) in the used samples. Expression of GPIIIa serves as loading control. The data is representative of three individual experiments (modified from Dütting et al., *J Thromb Haemost*, 2015).

2.1.4 Buffers and media

If not stated otherwise, all buffers were prepared in deionized water obtained from a MilliQ Water Purification System (Millipore, Schwalbach, Germany). pH was adjusted with HCl or NaOH.

Laemmli sodium dodecyl sulfate (SDS) sample buffer (4x)

Tris-HCl, pH 6.8	200 mM
Glycerol	40 %
Bromophenol blue (3',3",5',5"-tetra-bromophenol-sulfonphthalein)	0.04 %
SDS	8 %
β-mercaptoethanol (reducing conditions)	20 %

Laemmli running buffer for SDS-PAGE

Tris	0.25 M
Glycine	1.92 M
SDS	35 mM

Lysis buffer for platelet lysates, pH 8.0

Tris, pH 7.4	15 mM
NaCl	155 mM
EDTA	1 mM
NaN_3	0.005 %
IGEPAL CA-630	1 %
Protease inhibitor cocktail	1 %

Lysis buffer (2x) for tyrosine phosphorylation studies, pH 7.5

NaCl	300 mM
Tris	20 mM
EGTA	2 mM
EDTA	2 mM
NaF	10 mM
Na_3VO_4	4 mM
IGEPAL CA-630	1 %
Protease inhibitor cocktail	1 %

Phosphate-buffered saline (PBS), pH 7.14

NaCL	137 mM
KCL	2.7 mM
KH_2PO_4	1.5 mM
Na_2HPO_4 x H_2O	8 mM

PBS-T (Wash buffer for ELISA)

PBS (1 x)	
Tween	0.1 %

Separating gel buffer (SDS-PAGE), pH 8.8

Tris-HCl	1.5 M

Sodium citrate, pH 7.0

Sodium citrate	0.129 M

Stacking gel buffer (SDS-PAGE), pH 6.8

Tris-HCl	0.5 M

Stripping buffer (“mild”), pH 2.0

SDS	1 %
Glycine	25 mM
in PBS	

Tris-buffered saline (TBS), pH 7.3

Tris-HCl	20 mM
NaCl	137 mM

TBS-T (Wash buffer for Western blotting)

TBS (1x)	
Tween® 20	0.1 %

Tyrode-HEPES buffer, pH 7.4

NaCl	134 mM
Na_2HPO_4	0.34 mM
KCl	2.9 mM
$NaHCO_3$	12 mM
HEPES	5 mM
$MgCl_2$	1 mM
Glucose	5 mM
BSA	0.35 %

2.2 Methods

2.2.1 Platelet preparation and washing

Mice were bled under isoflurane anesthesia from the retro-orbital plexus. The blood was collected up to 1 ml into an Eppendorf tube containing 300 µl heparin (20 U/ml). 200 µl of heparin were added and blood was centrifuged at 300 g (Eppendorf Centrifuge 5415C) for 6 min at room temperature (RT). The supernatant and buffy coat were transferred into new Eppendorf tubes containing 200 µl heparin and centrifuged at 300 g for 6 min at RT. To obtain *platelet rich* plasma (prp) the supernatant and buffy coat were transferred without erythrocytes into new eppendorf tubes. To prepare washed platelets, the prp was centrifuged for 5 min at 800 g at RT in presence of *prostacyclin* (PGI_2) (0.1 µg/ml) and apyrase (0.2 U/ml) to prevent pre-activation of platelets. The obtained pellet was resuspended in 1 ml Ca^{2+}-free Tyrode's buffer containing PGI_2 and apyrase, incubated for 10 min at 37 °C and afterwards centrifuged for 5 min at 800 g at RT. After repeating the washing step once, platelet numbers were determined by analyzing a 1:1 dilution of platelet suspension with a Sysmex KX-21N automated hematology analyzer. Finally, the pellet was resuspended in an appropriate volume of Tyrode's buffer containing apyrase (0.02 U/ml) to reach the required amount of platelets for respective experiments. Washed platelets were rested for at least 30 min at 37 °C before platelet analysis.

2.2.2 Platelet counting

For determination of platelet size and platelet count of previously obtained washed platelet suspension, 50 µl of washed platelets were added to a volume of 50 µl Ca^{2+}-free Tyrode's buffer. Platelet count and size were determined with an automated hematology analyzer (Sysmex Corp).

2.2.3 Inhibitor-treatment of platelets

For characterizing effects of Rac1-inhibitors prepared platelets were supplied with the respective amount of either NSC23766, EHT1864 or vehicle (ddH_2O) and incubated for 5 min at 37 °C prior to the experiments.

2.2.4 Flow cytometric analysis

For determination of glycoprotein (GP) expression on platelet surface, washed platelets were obtained as described in 2.2.1 and adjusted to 0.5 x 10^5 platelets/µl by addition of Tyrode's buffer (+2 mM Ca^{2+}). Platelets were incubated in the presence of NSC23766 and EHT1864 for 5 min at 37 °C. A volume of 10 µl of the fluorophore-conjugated (FITC) monoclonal antibodies described in section 2.1.2 was mixed with 50 µl washed blood and stained 15 min at RT. The reaction was stopped by adding 500 µl of 1xPBS and samples were analyzed with a FACSCalibur Cell Analyzer (BD Biosciences).

To analyze platelet activation responses, washed platelets, pretreated with NSC23766 and EHT1864 were adjusted to a concentration of 0.5 x 10^5 platelets/µl in Tyrode's buffer (+ 2 mM Ca^{2+}). 50 µl of the suspension were added to FACS tubes and platelets were activated by 7 µl of appropriate agonists for 6 min at 37 °C followed by 6 min at RT in presence of 20 µl 1:1 mixture of phycoerythrin (PE)-coupled JON/A and fluorescein isothiocyanate (FITC)-coupled P-selectin antibodies. The reaction was stopped by adding 500 µl 1xPBS per tube. The samples were analyzed using a FACSCalibur Cell Analyzer (BD Biosciences) with Cell Quest™ software (BD Biosciences, Heidelberg, Germany).

2.2.5 Determination of phosphatidylserine exposure

Washed platelets were adjusted to a concentration of 0.5 x 10^5 platelets/µl in Tyrode's buffer (+ 2 mM Ca^{2+}) and treated with indicated concentrations of NSC23766 and EHT1864 for 5 min at 37 °C. 50 µl platelet suspension were incubated for 15 min at 37 °C in presence of DyLight 488-coupled Annexin V. The reaction was stopped by addition of Tyrode's buffer (+ 2 mM Ca^{2+}) and samples were analyzed immediately using FACSCalibur Cell Analyzer.

2.2.6 Aggregometry

To analyze the aggregation properties, washed platelets were prepared and adjusted to a concentration of 5 x 10^5 platelets/µl by adding Ca^{2+}-free Tyrode's buffer and apyrase (0.02 U/ml). 50 µl of platelet suspension were diluted with 110 µl of Tyrode's buffer (+ 2 mM Ca^{2+}). For ADP stimulation measurements, heparinized prp was used instead of washed platelets. Washed platelets were pretreated with NSC23766, EHT1864 or vehicle for 5 min at 37 °C and reaction was started by adding 1.6 µl of different agonists (100-fold concentrated) in presence of 70 µg/ml fibrinogen, except

when stimulating with thrombin. Light transmission was measured for 10 min on a Fibrintimer 4 channel aggregometer (APACT Laborgeräte und Analysesysteme). For calibration before measurement, 160 µl Ca^{2+}-free Tyrode's buffer (for washed platelets) or plasma (for prp) were set as 100 % aggregation and unstimulated washed platelet suspension or prp as 0 % aggregation.

2.2.7 Platelet spreading

2.2.7.1 Adhesion on human fibrinogen

Glass coverslips (24 x 60 mm) were coated with 100 µg/ml fibrinogen/PBS overnight at 4 °C and blocked with sterile 1 % BSA/PBS for at least 1 h at RT before starting the experiment. After blocking, the coverslips were washed with 300 µl PBS three times and rinsed with Tyrode's buffer (+ 2 mM Ca^{2+}) previously warmed up to 37 °C. 100 µl of washed platelets, previously adjusted to 0.3 x 10^5 platelets/µl and incubated with either NSC23766, EHT1864 or vehicle for 5 min at 37 °C, were added to the coverslips and stimulated with thrombin (0.01 U/ml). Platelets were allowed to spread on fibrinogen matrix for time periods of 5, 10, 15 and 30 min. The reaction was stopped by addition of 300 µl of 4 % PFA/PBS. Excessive liquid was removed after 5 min and the slips were covered with glass slides. Platelets were then visualized with a Zeiss Axiovert 200 inverted microscope (100x/1.4 oil objective) using differential interfering contrast (DIC) microscopy. Representative images were recorded with a CoolSNAP-EZ camera (Visitron) and analyzed offline with MetaVue software (Molecular Devices).

2.2.7.2 Adhesion on murine vWF

Glass coverslips (24 x 60 mm) were coated with 200 µl polyclonal rabbit-α-human vWF antibody (1:500 dilution in 50 mM carbonate-bicarbonate) overnight at 4 °C. Coverslips were rinsed with sterile PBS and blocked with 3 % BSA/PBS for at least 1 h at 37 °C. Coverslips were washed twice with 300 µl sterile PBS and coated with 200 µl previously obtained mouse plasma for 2 h at 37 °C. To remove remaining BSA, coverslips were washed with sterile PBS and covered with Tyrode's buffer (+ 2 mM Ca^{2+}), previously warmed to 37 °C. Heparinized washed platelets were adjusted to 0.3 x 10^6 platelets/µl with Ca^{2+}-free Tyrode's buffer and incubated with integrillin (60 µg/ml) and botrocetin (10 µg/ml) for 10 min. In the meantime NSC23766, EHT1864 or vehicle, respectively, were added and suspension was incubated for 5 min. 100 µl of platelet suspension were allowed to spread on vWF

matrix for indicated time periods at 37 °C. Reaction was stopped by addition of 300 µl of sterile 4 % PFA/PBS onto the coverslips. Excessive liquid was removed after 5 min and slips were covered with glass. Spread platelets were then visualized and analyzed as describes above.

2.2.8 Clot retraction

Mice were bled with heparin-free capillaries under isoflurane anesthesia from the retro orbital plexus and blood was collected into Eppendorf tubes containing 70 µl sodium citrate. Platelets were washed as described above except from using Ca^{2+}-free Tyrode's buffer instead of heparin. Afterwards they were adjusted to 0.3 x 10^6 platelets/µl with previously collected plasma. 250 µl of the obtained platelet suspension were pipetted into an aggregometry cuvette and 20 mM $CaCl_2$ and 1 µl red blood cells were added. After incubation with NSC23766, EHT1864 or vehicle for 5 min at 37 °C, platelet clotting was induced by addition of thrombin (3 U/ml) (Sigma) and cuvettes were incubated at 37 °C. The clot retraction was recorded with a digital camera at indicated time points over a time span of 4.5 h after activation. To analyze the retraction ability, the volume of leftover fluid in the cuvette was measured. The starting volume of 250 µl platelet suspension was set as 100 %.

2.2.9 Protein phosphorylation studies

Washed platelets were obtained as previously described, except for using Tyrode's buffer without BSA for the last washing steps. Platelets were adjusted to 5 x 10^5 platelets/µl and stimulated with 0.1 U/ml thrombin under constant stirring conditions at 37 °C in the presence of 2 U/ml apyrase, 10 µM indomethacin and 2 mM EDTA. At respective time points the reaction was stopped by 1:1 addition of ice-cold lysis buffer. Samples were lysed for 20 min at 4 °C and afterwards centrifuged at 14,000 rpm for 10 min at 4 °C. The supernatant was collected, and after addition of 4x Laemmli buffer and β-Mercaptoethanol for reducing conditions samples were boiled at 95 °C for 5 min. Next, samples were analyzed by western blotting.

2.2.10 SDS-PAGE and Western Blot

For separation of proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 15-25 µl per sample were loaded onto a previously prepared gel with 4 % stacking part and 12 % separating part, provided in a gel

chamber filled with Laemmli buffer. Proteins were separated at 20-30 mA for approximately 1.5 h and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in BSA or fat-free milk dissolved in TBS-T at least 1 h at RT or overnight at 4 °C. The blocked membrane was then incubated with respective primary antibody overnight at 4 °C. Membranes were washed three times in TBS-T for 10 min at RT. Afterwards membranes were incubated with appropriate HRP-labeled secondary antibody for 1 h at RT. After extensive washing with TBS-T the proteins were visualized by ECL using X-ray developing films.

2.2.11 Data analysis

Results from at least 3 independent experiments per group are presented as mean ± SD. Differences between two groups were assessed by ANOVA with Dunnett's T3 as post hoc test GraphPad Prism. *p*-values < 0.05 were considered statistically significant (*), $p < 0.01$ (**) and $p < 0.001$ (***).

3 Results

3.1 Titration of NSC23766 and EHT1864 in *Rac1*$^{-/-}$ and *Rac1*$^{+/+}$ mouse platelets using flow cytometry

3.1.1 NSC23766 and EHT1864 exhibit dose-dependent inhibition in *Rac1*$^{+/+}$ mouse platelets

To date, different concentrations of NSC23766 and EHT1864 have been used in experimental settings, regardless of the established IC_{50} value (50 µM) for both compounds[38,50–52,59]. Based on these reported IC_{50} values, the following experiment was designed to establish a suitable concentration for functional inhibition of Rac1 in murine platelets. In a first set of experiments, washed murine *Rac1*$^{+/+}$ platelets were treated with different concentrations of NSC23766 (low: 10, 50; intermediate: 100; high: 300 µM), EHT1864 (low: 10, 50; intermediate: 100; high: 200 µM) or as control the respective amount of solvent (ddH_20) prior to stimulation of GPCRs or (hem)ITAM signaling pathways. By using flow cytometry, platelet activation was measured by levels of active αIIbβ3 (integrin activation) and exposed P-selectin (α-granule secretion). Active αIIbβ3 was labeled with JON/A-PE antibody which binds with high affinity to the active conformation of the integrin[60], whereas anti P-selectin-FITC antibody was used to monitor the degranulation-dependent P-selectin surface expression (Figure 3-1).

In NSC23766-treated *Rac1*$^{+/+}$ platelets, aIIbβ3 activation and α-granule secretion were decreased dose-dependently after stimulation of GPCRs with thrombin, as well as (hem)ITAM receptors, either stimulated with *collagen-related peptide* (CRP), a ligand of GPVI, or the snake venom toxin *rhodocytin*, a ligand of CLEC-2.

The inhibition was less pronounced after selective stimulation of the P2Y receptors with ADP or activation of the thromboxane (TP) receptors with the TxA_2 analogue U46619. In these experimental settings, APD and TxA_2 act as weak agonists and only induce integrin activation without concomitant P-selectin exposure. Co-stimulation of platelets with ADP and U46619 strongly potentiates platelet reactivity and was significantly inhibited by NSC23766 and EHT1864.

Comparable to the reported IC_{50} value for NSC23766 (50 µM), integrin activation was reduced by approximately 50 % upon stimulation with thrombin or CRP. Higher inhibitor concentrations significantly diminished (100 µM) or almost

abolished (300 µM) platelet activation after GPVI- and GPCR-stimulation. Similarly, α-granule secretion was reduced after stimulation with the respective agonists.
Likewise, *Rac1*$^{+/+}$ platelets were treated with indicated concentrations of EHT1864. A diminished αIIbβ3 activation and α-granule secretion after stimulation of GPCRs and (hem)ITAM receptors could be observed at all indicated inhibitor concentrations. Inhibition of platelet activation by EHT1864 appeared to be less potent than by NSC23766. Here, the previously established IC_{50} value (50 µM[38]) did not lead to an

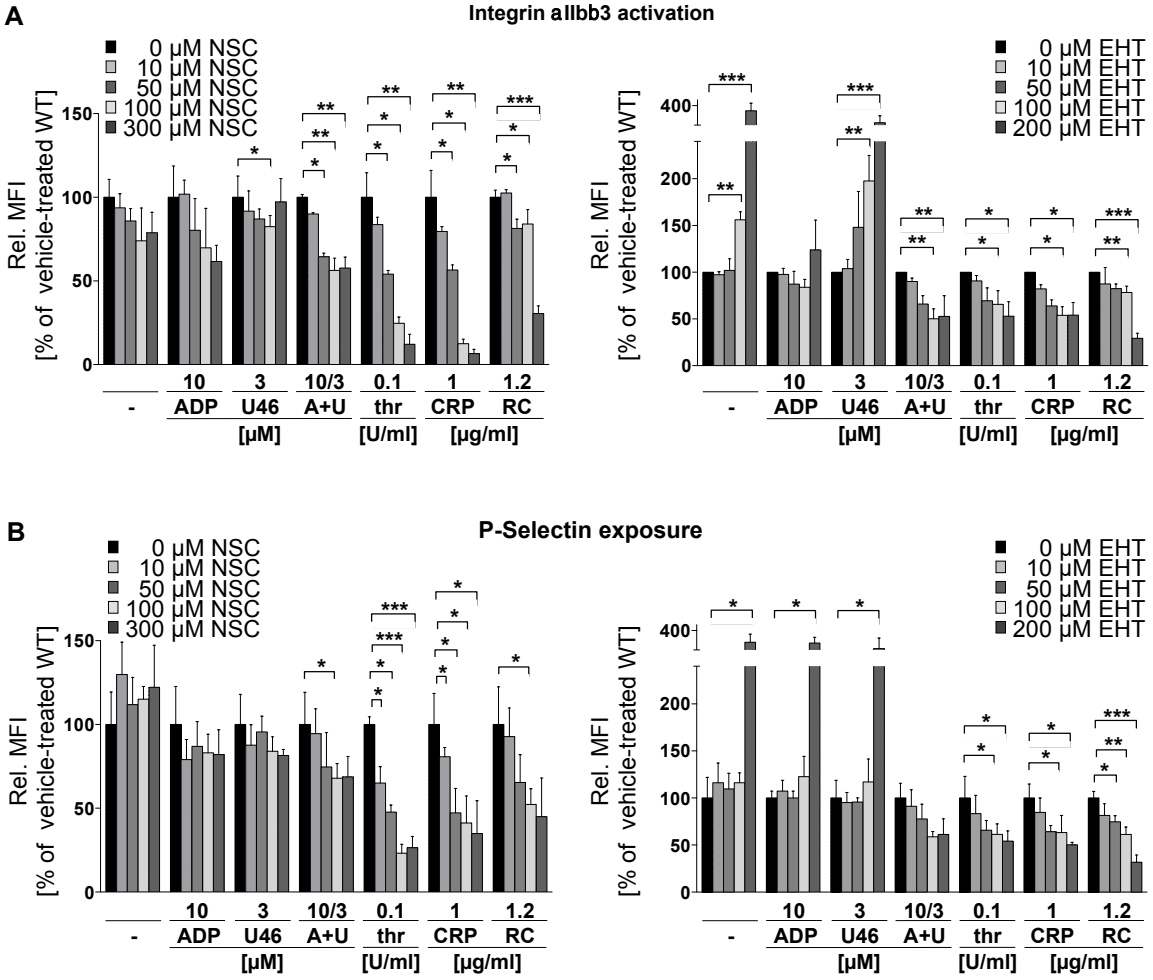

Figure 3-1 Dose-dependent inhibition of αIIbβ3 activation and α-granule release by NSC23766 (NSC) and EHT1864 (EHT) in *Rac1*$^{-/-}$ platelets. Flow cytometric analysis of αIIbβ3 activation (A) and degranulation dependent P-selectin exposure (B) in response to the indicated agonists in washed *Rac1*$^{-/-}$ platelets after treatment with different concentrations of NSC23766, EHT1864 or vehicle for 5 min. Results are relative mean fluorescence intensities (MFIs) normalized to vehicle-treated wild-type (WT) control ± standard deviation of four mice per group, and are representative of three individual experiments. *Significant difference ($P < 0.05$), **Significant difference ($P < 0.01$), ***Significant difference ($P < 0.001$) as compared with vehicle-treated control group (modified from Dütting *et al., J Thromb Haemost*, 2015).

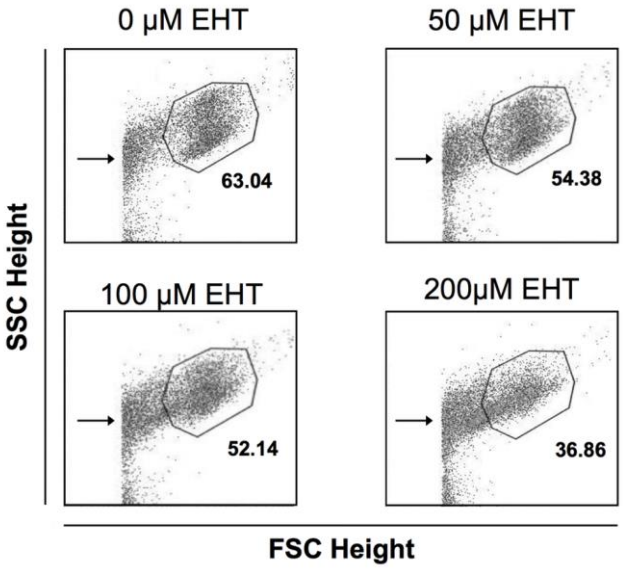

Figure 3-2: Increased cell debris formation in *Rac1*$^{+/+}$ platelets at resting stage after treatment with EHT1864 (EHT). Representative dot plots showing side scatter (SSC) and forward scatter (FSC) from flow cytometric analysis of *Rac1*$^{+/+}$ platelets after treatment with different concentrations of EHT1864 (EHT) or vehicle for 5 min. Numbers indicate events (in %) within the standard gate for vital mouse platelet population. Arrows indicate site of cell debris. Data is representative of three individual experiments.

adequate inhibition of platelet activation, in contrast to application of intermediate doses (100 µM). Importantly, increased signal for integrin activation and P-selectin exposure was observed at resting stage after treatment with a high dose (200 µM) of EHT1864. *Forward scatter* (FSC) and *side scatter* (SSC) characteristics of resting platelets were significantly altered under these conditions, and the presence of cell debris could be identified (Figure 3-2).

In conclusion, these data demonstrate the ability of NSC23766 and EHT1864 to inhibit αIIbβ3 integrin activation and α-granule secretion in *Rac1*$^{+/+}$ platelets. In addition to its properties as a potent Rac1 inhibitor, higher doses of EHT1864 seemed to induce spontaneous platelet activation and cell death.

3.1.2 NSC23766 and EHT1864 exhibit Rac1-independent effects in *Rac1*$^{-/-}$ mouse platelets

NSC23766 has been established as a selective inhibitor of Rac1 only, whereas EHT1864 blocks Rac1, Rac1b, Rac2 and Rac3 activity. In mouse platelets, Rac1 is the only isoform expressed on protein level[30].

To further determine possible off-target effects of NSC23766 and EHT1864, *in vitro* titration experiments were performed in the absence of Rac1 using platelets isolated

from mice with platelet-specific Rac1-deficiency. The generation of these knockout mice was described before[31].

In line with previous findings[30,33], *Rac1*$^{-/-}$ platelets exhibited defective signaling upon (hem)ITAM stimulation. Application of NSC23766 and EHT1864 did not significantly alter activation after stimulation with CRP and rhodocytin (Figure 3-3). In sharp contrast, both inhibitors significantly impaired the activation of *Rac1*$^{-/-}$ platelets after stimulation of PARs with thrombin. Indeed, integrin activation and α-granule secretion

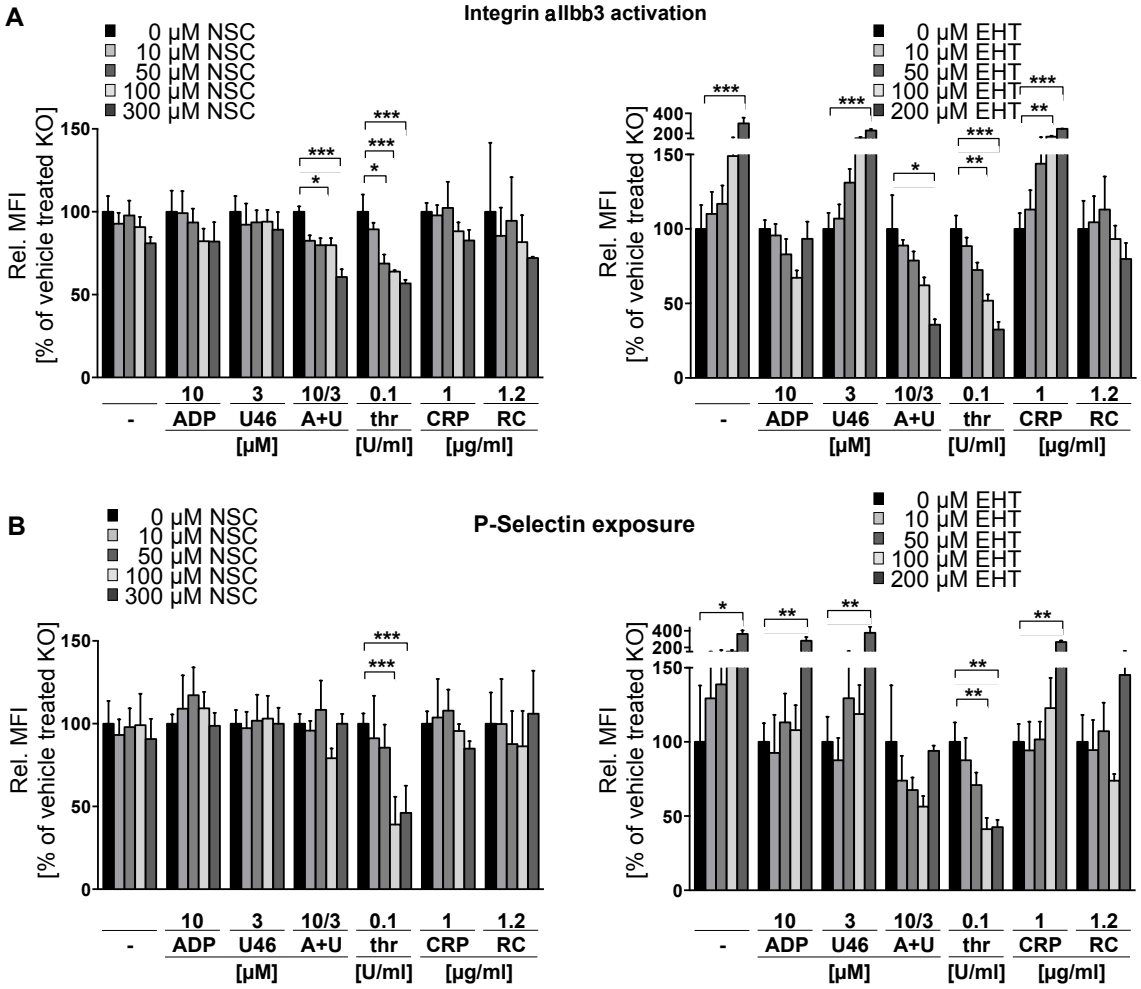

Figure 3-3 Dose-dependent inhibition of αIIbβ3 activation and α-granule release by NSC23766 (NSC) and EHT1864 (EHT) in *Rac1*$^{-/-}$ platelets. Flow cytometric analysis of αIIbβ3 activation (A) and degranulation dependent P-selectin exposure (B) in response to the indicated agonists in washed *Rac1*$^{-/-}$ platelets after treatment with different concentrations of NSC23766, EHT1864 or vehicle for 5 min. Results are relative mean fluorescence intensities (MFIs) normalized to vehicle-treated wild-type (WT) control ± standard deviation of four mice per group, and are representative of three individual experiments. *Significant difference ($P < 0.05$), **Significant difference ($P < 0.01$), ***Significant difference ($P < 0.001$) as compared with vehicle-treated control group (Dütting *et al., J Thromb Haemost*, 2015).

were reduced after application of low (50 µM; $p < 0.5$), intermediate (100 µM; $p < 0.1$) and high inhibitor concentrations (200/300 µM; $p < 0.01$). Both compounds similarly impaired activation by co-stimulation of P2Y-receptors and the TP-receptors.

In addition, EHT1864-treated *Rac1*$^{-/-}$ platelets showed, similarly as observed in *Rac1*$^{+/+}$, increased MFIs of active αIIbβ3 and P-selectin in absence of external stimulation, as well as similar alternations in FSC and SSC characteristics with increased amount of cell debris. This indicates that high doses of EHT1864 might have destructive effects on platelets independently of Rac1 inhibition.

These results clearly demonstrate that both compounds have Rac1-independent functions in mouse platelets, even at intermediate concentrations (100 µM).

3.2 NSC23766 and EHT1864 significantly affect surface expression of receptors and platelet viability

In the above presented data, Rac1-independent effects of inhibitors were observed in both wild-type and murine *Rac1*$^{-/-}$ platelets. Particularly, application of higher concentration of EHT1864 significantly increased platelet reactivity without agonist stimulation. Therefore, we further analyzed effects on platelet viability and surface expression of major platelet receptors upon NSC23766 and EHT1864 treatment.

3.2.1 EHT1864 triggers platelet apoptosis

Increased fluorescent intensities in cell sorting in unstimulated platelets can be a

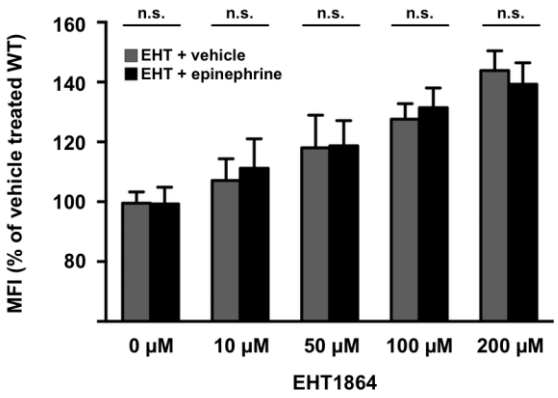

Figure 3-4: Integrin αIIbβ3 activation induced by EHT1864 is not a result of increased platelet procoagulant activity. Flow cytometric analysis of αIIbβ3 activation (JON/A) in WT platelets treated with EHT1864 and after co-stimulation with epinephrine. Platelets do not exhibit enhanced activation in response to epinephrine. n.s., not significant ($P > 0.05$).

result of enhanced platelet procoagulant activity and/or cell death, triggered by EHT1864. To test whether EHT1864 induced platelet activation and thereby PS exposure, platelets were treated with epinephrine in addition to the inhibitor. Epinephrine alone does not induce full activation of platelets, it rather potentiates persistent platelet activation by interaction with α2 adrenergic receptors[62]. However, *Rac1*$^{+/+}$ platelets did not show increased integrin αIIbβ3 activation after co-treatment with EHT 1864 and epinephrine compared to platelets treated solely with the inhibitor at all tested concentrations (Figure 3-4). These results indicate that integrin αIIbβ3 activation induced by high concentrations of EHT1864 was not due to increased platelet procoagulant activity, but more likely a result of cell destruction after treatment with the inhibitor.

To therefore define if integrin activation might be due to platelet apoptosis, the exposure of *phosphatidylserine* (PS) was measured. PS is a phospholipid component of the cell membrane normally restricted to the cytosolic side. Translocation of PS from extracellular membrane side to the cytosolic side is mediated by the ATP-dependent transporter enzyme flippase. In case of apoptosis or increased procoagulant activity, the flippase fails to control translocation of PS, which becomes exposed to the membrane surface[61]. Accumulation of PS on the outer layer of the plasma membrane can be detected in flow cytometry by measuring Annexin V binding.

Both, *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ platelets exposed slightly increased Annexin-V-DyLight488-

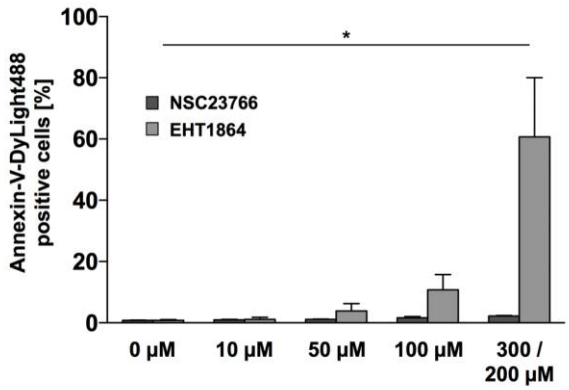

Figure 3-5: Dose-dependent increase of PS exposure in *Rac1*$^{+/+}$ platelets by EHT1864 but not NSC23766. Flow cytometric analysis of PS exposure in washed *Rac1*$^{+/+}$ platelets in response to EHT1864 and NSC23766. *Significant difference ($P < 0.05$) as compared with vehicle-treated control.

positive cells after treatment with low (50 µM) and intermediate (100 µM) concentrations of EHT1864, while high concentrations of the drug increased the ratio of PS-positive cells up to 69.8 ± 6 % (Figure 3-5). By contrast, treatment with vehicle (ddH_2O) or NSC23766 did not impair PS exposure at any tested concentration.

In anuclear platelets apoptosis induces a decrease of mitochondrial membrane depolarization ($\Delta\Psi_m$). During apoptosis the membrane potential of mitochondria cannot be maintained and depolarization can be observed. Investigation of mitochondrial membrane depolarization ($\Delta\Psi_m$) was performed in collaboration with the laboratory of R. Ahmadian (Institute of Biochemistry and Molecular Biology II, Medical Faculty of the Heinrich-Heine-University, Düsseldorf, Germany) and confirmed significant platelet apoptosis in *Rac1*$^{+/+}$ platelets in response to 100 µM and 200 µM EHT1864 (Data from Amin, Zhang, Ahmadian, not shown)[63]. Taken these results together with the presented dot plots in Figure 3-2 our results suggest that high doses of EHT1864 trigger apoptosis in murine platelets.

3.2.2 NSC23766 induces platelet receptor down-regulation

To investigate whether NSC23766 and EHT1864 have effects on the GP surface expression levels, washed platelets were incubated with inhibitors for 5 min, stained subsequently with antibodies against the major platelet receptors and analyzed by flow cytometry. *Rac1*$^{+/+}$ platelets exhibited significantly reduced expression levels of GPIb, V, IX, VI and the integrins α□, β1 and αIIbβ3 after application of NSC23766 in a dose-dependent manner (Table 3-1A). While low concentrations significantly impaired the expression only of integrin β1, severe down-regulation of all tested surface GPs could be observed after treatment with high concentrations. In comparison, *Rac1*$^{-/-}$ platelets that were only treated with vehicle exposed similar GP expression levels as similarly treated *Rac1*$^{+/+}$ platelets. Likewise, NSC23766 treatment induced reduction of GP surface expression in *Rac1*$^{-/-}$ platelets (Table 3-1B). This demonstrates that the impairment of GP expression does not occur due to Rac1-deficiency, but is induced by Rac1-independent effects of NSC23766. To test if EHT1864 similarly affects expression of the membrane GPs, experiments were repeated with EHT1864-treated *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ platelets. Glycoprotein surface expression was not significantly altered at any tested concentration of EHT1864 (Table 3-2).

Table 3-1: Dose-dependent reduction in platelet glycoprotein (GP) surface expression of (A) *Rac1*$^{+/+}$ and (B) *Rac1*$^{-/-}$ platelets by NSC23766 (NSC).

A

Rac1$^{+/+}$				
–	0 µM NSC	50 µM NSC	100 µM NSC	300 µM NSC
GPVI	56 ± 3	51 ± 3	51 ± 1	44 ± 2**
α2	73 ± 5	74 ± 2	71 ± 1	68 ± 2
β1	120 ± 4	103 ± 3**	100 ± 3*	83 ± 4***
CD9	1075 ± 22	1028 ± 30	985 ± 28	820 ± 17***
GPIb	305 ± 5	297 ± 9	290 ± 5	271 ± 6**
GPIX	362 ± 14	343 ± 6	328 ± 4	294 ± 4**
αIIbβ3	533 ± 25	504 ± 19	478 ± 20	416 ± 18**
GPV	266 ± 13	242 ± 8	226 ± 1*	199 ± 4**

B

Rac1$^{-/-}$				
–	0 µM NSC	50 µM NSC	100 µM NSC	300 µM NSC
GPVI	51 ± 4	53 ± 2	49 ± 3	42 ± 1***
α2	72 ± 2	71 ± 3	73 ± 1	70 ± 2
β1	124 ± 2	110 ± 3*	102 ± 2*	89 ± 2***
CD9	1099 ± 18	1032 ± 21	975 ± 28	810 ± 12**
GPIb	299 ± 5	302 ± 4	292 ± 2	265 ± 7**
GPIX	374 ± 16	353 ± 5	322 ± 7	282 ± 2***
αIIbβ3	541 ± 10	520 ± 13	465 ± 15*	432 ± 16*
GPV	252 ± 13	246 ± 4	221 ± 10*	194 ± 2**

Surface expression of platelet GPs was determined by flow cytometry. Washed platelets were incubated with vehicle or different concentrations of NSC23766, and then incubated with FITC-labeled antibodies under saturating conditions for 15 min at RT. Data are expressed as mean fluorescence intensity ± standard deviation (n=4), and are representative of three individual experiments. *Significant difference ($P < 0.05$), **Significant difference ($P < 0.01$), ***Significant difference ($P < 0.001$) as compared with vehicle-treated control (Dütting *et al., J Thromb Haemost*, 2015).

Table 3-2: No significant alternation in platelet glycoprotein (GP) surface expression of *Rac1*$^{+/+}$ in response to EHT 1864

Rac1$^{-/-}$				
–	0 µM EHT	50 µM EHT	100 µM EHT	200 µM EHT
GPVI	49 ± 4	44 ± 9	41 ± 3	40 ± 4
α2	96 ± 7	76 ± 11	73 ± 4	70 ± 6
β1	117 ± 1	126 ± 6	119 ± 9	112 ± 6
CD9	1200 ± 31	1190 ± 9	1150 ± 18	1141 ± 21
GPIb	260 ± 10	255 ± 7	250 ± 7	244 ± 3
GPIX	368 ± 12	372 ± 4	354 ± 3	343 ± 5
αIIbβ3	600 ± 30	582 ± 11	575 ± 21	569 ± 12
GPV	259 ± 41	239 ± 12	240 ± 9	241 ± 14

Expression of GPs on the platelet surface was determined by flow cytometry. Washed platelets were incubated with vehicle or different concentrations of EHT1864, and then incubated with FITC-labeled antibodies under saturating conditions for 15 min at RT. Data are expressed as mean fluorescence intensity ± standard deviation (n=4), and are representative of three individual experiments.

These findings reveal significant (off)-target effects of NSC23766 and EHT1864. Since most sufficient inhibition of Rac1-mediated platelet function was observed upon drug doses of 100 µM, while observable off-target effects were kept to a minimum, the inhibitor concentration of 100 µM was used for both compounds for all subsequent experiments.

3.3 Rac1 inhibitors impair inside-out signaling of platelets

To analyze and directly compare the efficacy of NSC23766 and EHT1864 on agonist-

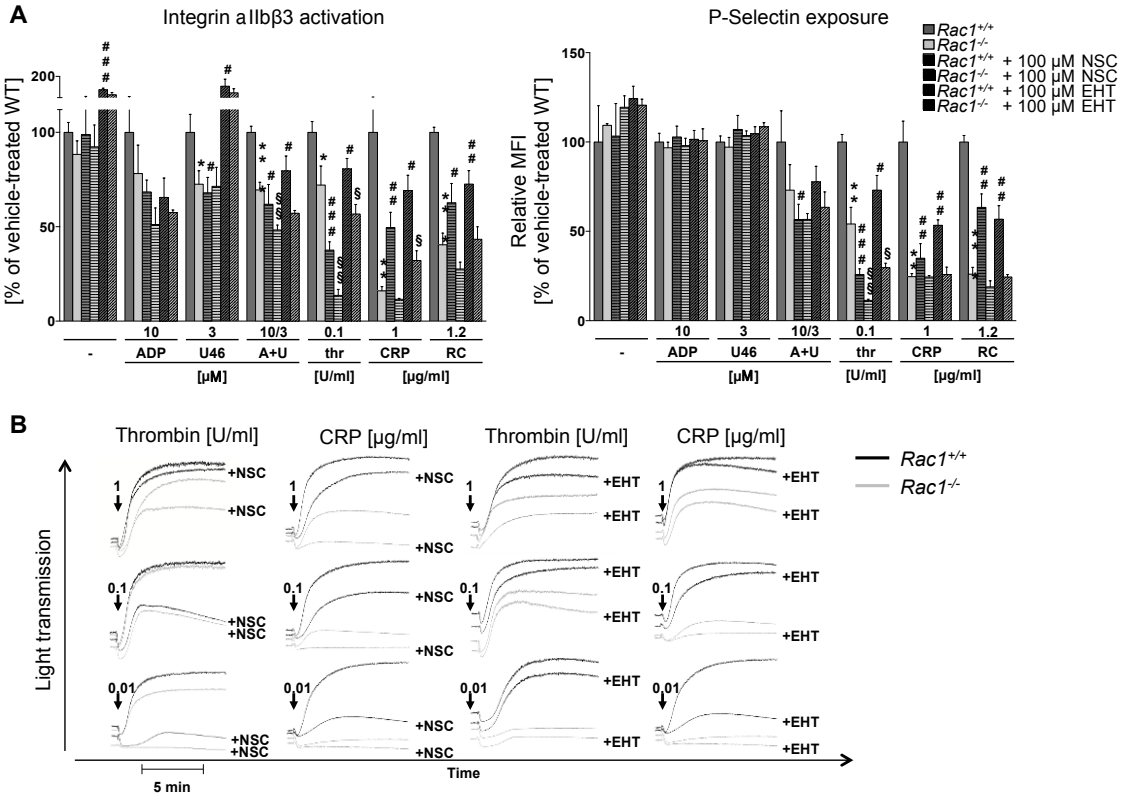

Figure 3-6: Rac1-independent effects of NSC23766 (NSC) and EHT1864 (EHT) on integrin inside-out signaling and platelet aggregation after platelet activation. (A) Flow cytometric analysis of αIIbβ3 activation and degranulation-dependent P-selectin exposure in response to the indicated agonists in washed *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ platelets after treatment with 100 µM NSC23766, EHT1864 or vehicle for 5 min. Results are relative mean fluorescence intensities (MFI) normalized to vehicle-treated wild-type (WT) control ± standard deviation of four mice per group, and are representative of three individual experiments. **(B)** Washed platelets from *Rac1*$^{+/+}$ (black line) and *Rac1*$^{-/-}$ (gray line) mice were incubated for 5 min prior to the experiment with NSC23766 or EHT1864, and then activated with the indicated concentrations of thrombin or CRP; light transmission was recorded on a Fibrintimer four-channel aggregometer. Representative aggregation traces of at least three individual experiments are depicted. *Significant difference ($P < 0.05$) between knockout and vehicle-treated WT control. #Significant difference ($P < 0.05$) between inhibitor-treated WT and vehicle-treated WT control. §Significant difference ($P < 0.05$) between inhibitor-treated knockout and vehicle-treated knockout control. CRP: collagen-related peptide; RC: rhodocytin (Dütting *et al.*, *J Thromb Haemost*, 2015).

induced platelet activation in the presence or absence of Rac1, washed platelets isolated from *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ mice were incubated with either 100 µM NSC23766, 100 µM EHT1864 or vehicle (ddH_2O). Afterwards, active αIIbβ3 and P-selectin surface exposure in response to external receptor stimulation was determined by flow cytometry.

Both, NSC23766 and EHT1864 reduced αIIbβ3 and α-granule release in response to stimulation with CRP in *Rac1*$^{+/+}$ platelets almost comparable to the levels seen in vehicle-treated *Rac1*$^{-/-}$ platelets. NSC23766 appears to have stronger inhibitory potential over EHT1864 (A).

In literature, the role of Rac1 in GPCR signaling of murine platelets is controversially discussed[30,33,42]. Here, a remarkable GPCR signaling defect was observed in Rac1-deficient platelets after receptor stimulation (Figure 3-6A). In line with this, NSC23766 and EHT1864 also blocked αIIbβ3 and α-granule release in wild-type platelets in response to GPCR stimulation with thrombin as well as ADP, U46619 and co-stimulation. Inhibition was almost comparable to the vehicle-treated *Rac1*$^{-/-}$ platelets, however, similar to inhibition in *Rac1*$^{+/+}$ platelets, NSC23766 was more potent compared to EHT1864. Both inhibitors showed significant additional inhibitory effects on thrombin-induced activation in *Rac1*$^{-/-}$ platelets.

To further investigate whether the defective αIIbβ3 activation and α-granule release in the presence of NSC23766 and EHT1864 affects platelet aggregation, *ex vivo* aggregation responses were analyzed after thrombin and CRP stimulation (Figure 3-6B). Aggregation of *Rac1*$^{-/-}$ platelets was partially impaired in response to GPCR stimulation. While application of EHT1864 decreased aggregation response upon agonist stimulation, NSC23766 virtually abolished aggregation of *Rac1*$^{+/+}$ platelets. Treatment of *Rac1*$^{-/-}$ platelets with NSC23766 or EHT1864 further decreased the defective aggregation response to GPCR and ITAM signaling. The potency of inhibition by NSC23766 and EHT1864 could be suppressed by inducing platelet aggregation with high agonist concentrations.

These results clearly confirm a role for Rac1 as a mediator of GPCR signaling. NSC23766 and EHT1864 lead to defective (hem)ITAM and GPCR signaling in *Rac1*$^{+/+}$ platelets and moreover enhance signaling defects in *Rac1*$^{-/-}$ platelets, which could be confirmed by *ex vivo* aggregation studies.

3.4 Rac1 inhibitors impair outside-in signaling of platelets

Ligand-occupied integrin αIIbβ3 triggers integrin outside-in signaling and leads to cytoskeletal reorganization and platelet spreading[12,64]. It has been proposed that Rac1 is crucial for platelets to form lamellipodia and to spread on fibrinogen-coated surface upon stimulation with thrombin, while they retain the ability to adhere and form filopodia[30,33]. In line with this, NSC23766 and EHT1864 have been reported to cause impaired platelet spreading in wild-type platelets comparable to the respectively used Rac1 knockout models[65,66].

To characterize the effects of NSC23766 and EHT1864 on αIIbβ3-mediated outside-in signaling, *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ platelets were allowed to spread on a fibrinogen-coated surface in the presence of low-concentrated thrombin (0.01 U/ml) in presence or absence of Rac1 inhibitors.

Treatment with vehicle did not change the ability of *Rac1*$^{+/+}$ platelets to adhere on the fibrinogen-coated surface and fully spread (Figure 3-7A). After treatment with NSC23766 or EHT1864, *Rac1*$^{+/+}$ platelets retained the ability to form filopodia; however, significantly less number of platelets spread fully. The lamellipodium formation of platelets was diminished and the spreading process was kinetically delayed in comparison to vehicle-treated platelets. NSC23766 and EHT1864 reduced the number of platelets that formed filopodia and lamellipodia by 20 % compared to

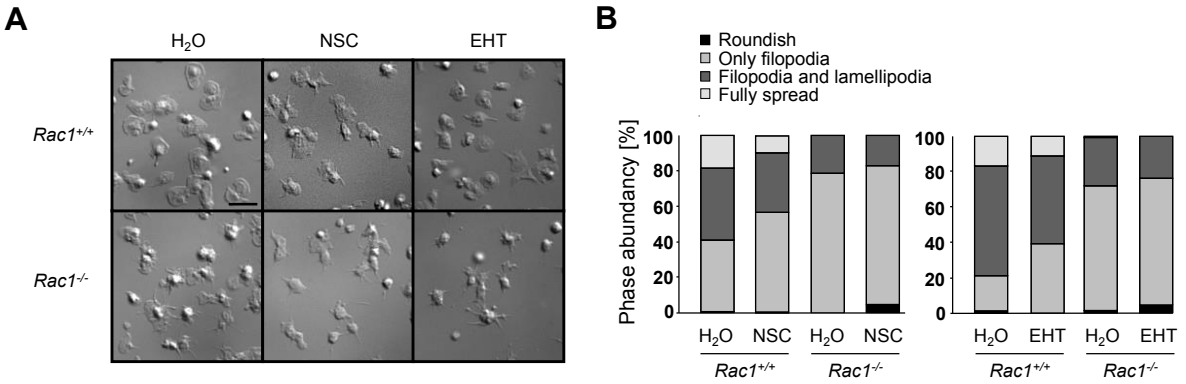

Figure 3-7: Specific inhibition of αIIbβ3-mediated spreading. (A, B) Washed platelets from *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ mice were allowed to spread on fibrinogen (100 µg/ml) for 30 min after stimulation with 0.01 U/ml thrombin. Prior to the experiment, platelets were incubated with 100 µM NSC23766, EHT1864 or vehicle for 5 min. **(A)** Representative differential interference contrast images of two individual experiments and **(B)** statistical evaluation of the percentages of spread platelets at different spreading stages are shown. 1, roundish; 2, only filopodia; 3, filopodia and lamellipodia; 4, fully spread. Scale bar = 5 µM (Dütting *et al., J Thromb Haemost*, 2015).

vehicle-treated *Rac1*$^{+/+}$ platelets (Figure 3-7B). It is known that Rac1 is crucial for lamellipodia formation and in line with this vehicle-treated *Rac1*$^{-/-}$ platelets lacked the ability to form lamellipodia and spread fully. Interestingly, these Rac1-deficient platelets retained ability to form filopodia; however, after application of NSC23766 and EHT1864 filopodia formation was less pronounced (Figure 3-7A-B).

Together, these data reveal that NSC23766 and EHT1864 diminish Rac1-mediated lamellipodia formation, but do not sufficiently prevent platelets from full spreading. Moreover, both inhibitors exhibit Rac1-independent effects in fibrinogen-mediated outside-in signaling, as filopodia formation is affected.

3.4.1 Minor contribution of NSC23766 in clot retraction

Integrin αIIbβ3 outside-in signaling also regulates clot retraction[67], which is essential for wound healing. Previous studies have indicated that Rac1 might be involved in this process. To analyze the effects of NSC23766 and EHT1864 during clot retraction, clot formation in *platelet-rich plasma* (prp) was induced by addition of thrombin. The subsequent response of the formed clot was monitored over time and the leftover fluid was measured. In marked contrast to previous findings[66,68], *Rac1*$^{-/-}$ platelets similarly retained the ability of clot retraction in comparison to *Rac1*$^{+/+}$ platelets (Figure 3-8). However, retraction kinetics were slightly delayed in *Rac1*$^{-/-}$ samples. Interestingly, treatment with EHT1864 minimally reduced retraction volumes in both, *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ platelets, whereas NSC23766 did not affect this

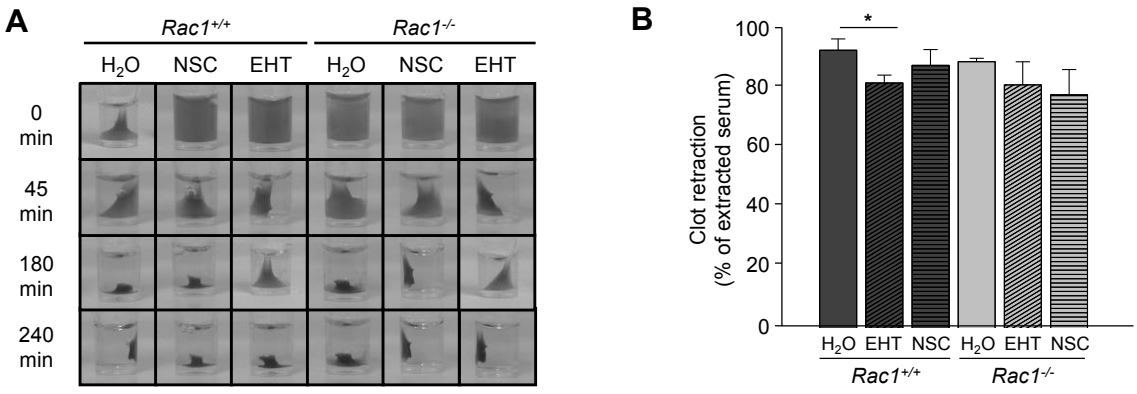

Figure 3-8: Minor off-target effects of EHT1864 (EHT) during clot retraction. (A, B) Clot retraction of platelet-rich plasma (prp) upon activation with 3 U/ml thrombin in the presence of 20 mmol/l $CaCl_2$ at the indicated time points. Representative images of three individual experiments **(A)** and statistical evaluation of clot retraction **(B)** are shown. *Significant difference ($P < 0.05$) as compared with vehicle control (modified from Dütting *et al.*, *J Thromb Haemost*, 2015).

process. Both inhibitors decelerated retraction kinetics similarly as observed in vehicle-treated *Rac1*$^{-/-}$ platelets. These results demonstrated that Rac1 plays only a minor role in clot retraction. In contrast to NSC23766, EHT1864 displayed an off-target effect on integrin αIIbβ3 outside-in-signaling.

3.5 Inhibitory role of NSC23766 in GPIb-mediated platelet spreading

Thrombus formation at sites of vascular injury is initiated by interaction of platelets with components of the exposed ECM, a process called tethering. This transient adhesion of platelets is mediated by vWF, which is immobilized on the ECM, and the platelet surface receptor GPIb.

Therefore we investigated platelet response upon adhesion on a vWF-coated surface under conditions of integrin αIIbβ3 blockade and enhanced binding activity by application of the snake venom toxin *botrocetin*[69]. In this experimental condition GPIb activation induces a cytoskeletal shape change, but the process is limited to contraction of the cell body and formation of long thin filopodia[4,70].

In line with previously published reports[71,72], *Rac1*$^{-/-}$ platelets showed remarkably impaired shape change and filopodia formation on vWF in comparison to *Rac1*$^{+/+}$ platelets (Figure 3-9). Interestingly, application of NSC23766 dramatically impaired filopodia formation in *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ platelets to a higher extend than it was observed in vehicle-treated *Rac1*$^{-/-}$ platelets. EHT1864 on the other hand showed milder reduction of filopodia formation compared to vehicle-treated *Rac1*$^{+/+}$ platelets.

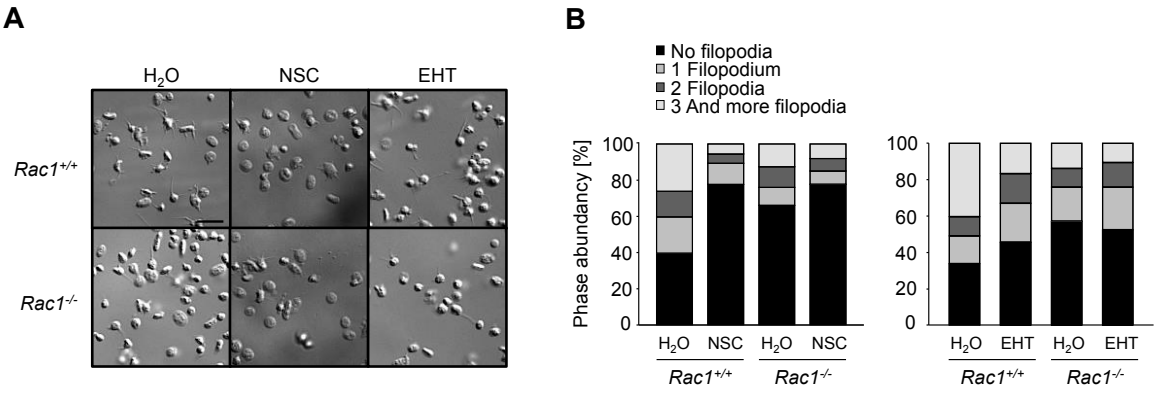

Figure 3-9: NSC23766 (NSC) inhibits glycoprotein Ib-mediated filopodia formation independently of Rac1. (A, B) Washed platelets of *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ mice were allowed to adhere and spread on von Willebrand factor for 20 min after incubation with 100 µM NSC23766, EHT1864 (EHT) or vehicle. Representative images of three individual experiments **(A)** and statistical evaluation of the percentage of filopodia-forming platelets **(B)** are shown (Dütting *et al., J Thromb Haemost*, 2015).

Of note, the number of adhered platelets on vWF-coated surface was decreased upon treatment with EHT1864, but not NSC23766.

These results demonstrate that NSC23766 has additional off-target effects on GPIb-mediated signaling in mouse platelets.

3.6 NSC23766 and EHT1864 do not inhibit GTPase activity of Cdc42 and RhoA

Rac1 is the only isoform of the Rac-family which is expressed on protein level in mouse platelets[30]. Two other major members of the family of Rho GTPases, namely Cdc42 (70.83% identity of amino acid composition) and RhoA (56.19% identity) are highly homologous to Rac1[26]. In platelets, these GTPases are expressed on a high level and might be targeted by NSC23766 and EHT1864 in absence of Rac1, thereby causing the described off-target effects. Previous works showed by *in vitro* GST-Rac1 pulldown assay[38] and *in vivo* Rho-GTP formation pulldown analyses[52], that NSC23766 and EHT1864 do not affect the nucleotide exchange of Cdc42 and RhoA. However, the inhibitors may affect activation in other ways than nucleotide exchange. Furthermore, NSC23766 only inhibits Rac1 interaction with the GEFs Trio and Tiam, but not Vav1, which as well interacts with RhoA. Thus, the inhibition of Rac1 might even enhance activation of RhoA by enhanced Vav1-interaction.

To analyze if NSC23766 and EHT1864 may affect Cdc42 and RhoA activity negatively or positively, washed platelets were pre-treated with NSC23766 and EHT1864 and stimulated with thrombin (0.1 U/ml) or CRP (1 µg/ml). The reaction was stopped with lysis buffer and GTPase activity was analyzed by G-LISA. Cdc42

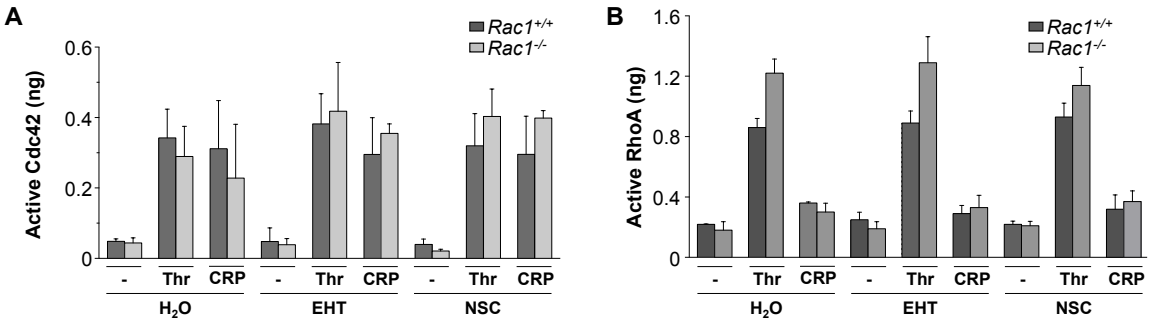

Figure 3-10: NSC23766 (NSC) and EHT1864 (EHT) do not interact with activity of Cdc42 and RhoA. Analysis of Cdc42 activity **(A)** and RhoA activity **(B)** in resting *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ platelets and after stimulation with either 0.1 U/ml thrombin or 1 µg/ml collagen-related peptide (CRP). Data is representative of 3 independent experiments. n.s., not significant (modified from Dütting *et al., J Thromb Haemost*, 2015).

and RhoA activity was not significantly impaired in *Rac1*$^{-/-}$ platelets upon stimulation with thrombin or CRP compared to *Rac1*$^{+/+}$. Moreover, the amount of active Cdc42 and active RhoA was not significantly altered after application of NSC23766 and EHT1864 (Figure 3-10).

To further test if Rac1 inhibitors potentially interfere with the functions of Cdc42 and RhoA independently of the nucleotide exchange, both inhibitors were applied to platelets isolated from mice with Rac1 and Cdc42 double knockout (referred to as *Rac1/Cdc42*$^{-/-}$)[31], or Rac1 and RhoA double knockout (referred to as *Rac1/RhoA*$^{-/-}$, Dütting, Nieswandt[58]) and platelet activation in response to different agonists was

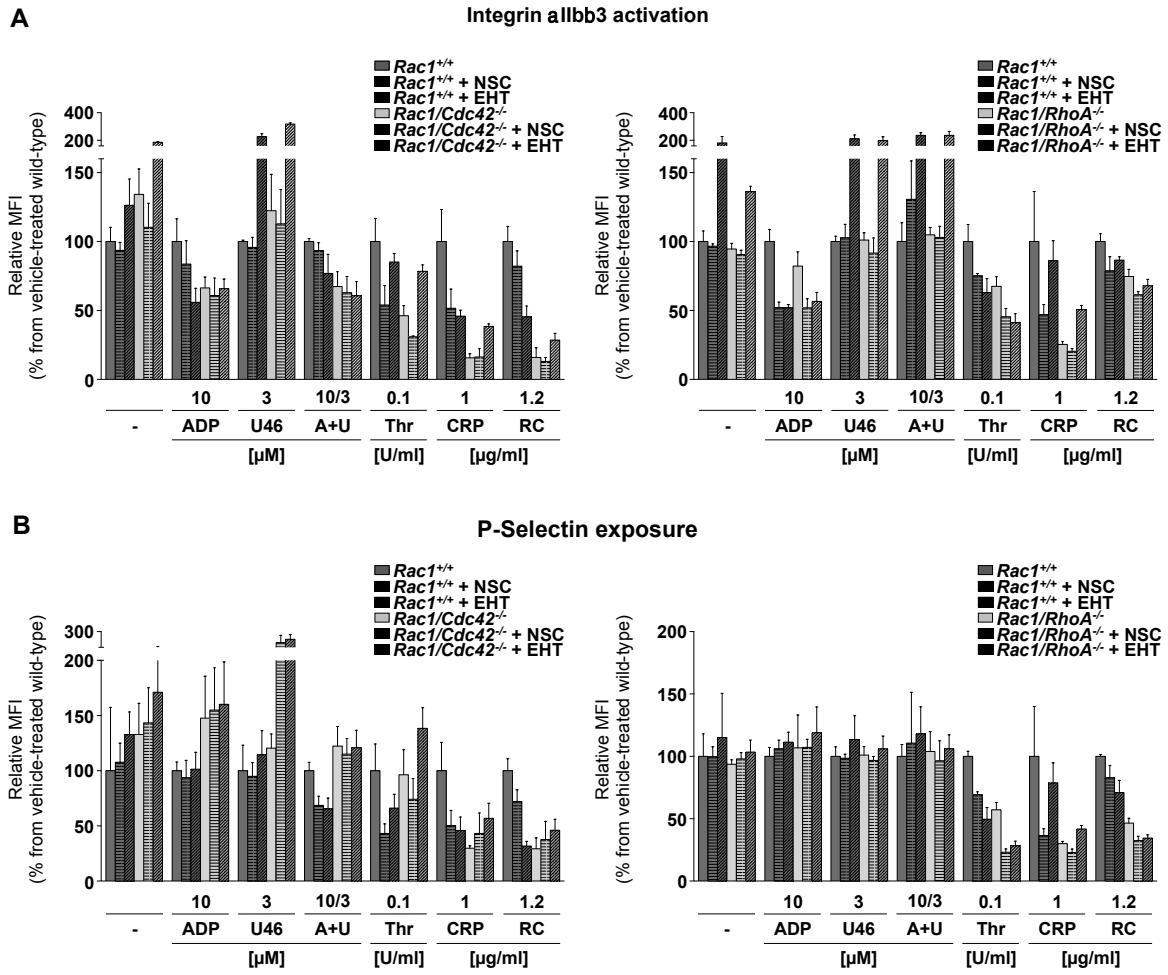

Figure 3-11: NSC23766 (NSC) and EHT1864 (EHT) do not act on Cdc42 and RhoA. Flow cytometry analysis of αIIbβ3 activation and degranulation dependent P-selectin exposure in response to the indicated agonists in washed *Rac1/Cdc42*$^{+/+}$, *Rac1/Cdc42*$^{-/-}$, *Rac1/RhoA*$^{+/+}$ and *Rac1/RhoA*$^{-/-}$ platelets after treatment with 100 µM NSC23766, EHT1864 or vehicle for 5 min. Results are presented as relative mean fluorescence intensities (MFI) normalized to vehicle-treated wild-type control ± standard deviation of four mice per group, and are representative of three individual experiments (modified from Dütting *et al*., *J Thromb Haemost*, 2015).

analyzed by flow cytometry.

Similar to the data obtained with *Rac1*$^{-/-}$ platelets, both NSC23766 and EHT1864 further reduced platelet activation in *Rac1/Cdc42*$^{-/-}$ platelets in response to thrombin as compared to vehicle-treated *Rac1/Cdc42*$^{-/-}$ (Figure 3-11). Consistently, *Rac1/RhoA*$^{-/-}$ platelets treated with NSC23766 or EHT1864 exposed remarkably impaired platelet activation in response to GPCR stimulation (Figure 3-11). It is important to note that the double-deficient platelets exposed significantly enhanced MFIs upon EHT1864-treatment in resting state and in response to GPVI stimulation, exceeding the MFIs in resting wild-type and Rac1-deficient platelets. In accordance with the data from 3.2.1 these findings show hypersensitive apoptotic response of *Rac1/Cdc42*$^{-/-}$ platelets to EHT1864.

These results strongly suggest that NSC23766 and EHT1864 do not inhibit Cdc42 and RhoA activity in addition to Rac1 in mice.

3.7 PAK1/PAK2 phosphorylation is inhibited independently of Rac1

By treatment with NSC23766, it has been shown that Rac1 mediates dephosphorylation of cofilin in human platelets by inhibiting the phosphorylation of the class II *p21 activated kinases* (PAK 4/5/6) after thrombin stimulation, whereas phosphorylation of class I PAKs (PAK1/PAK2) was unaltered[43]. Cofilin is a small actin dynamizing protein downstream of *LIM domain kinase 1* (LIMK1) and takes part

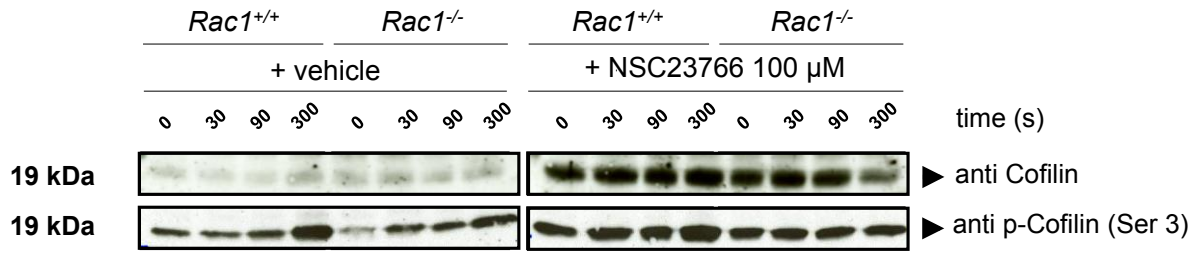

Figure 3-12: Dephosphorylation of cofilin is significantly affected by NSC23766. Washed platelets (7 x 10^5/µl) from *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ mice were incubated with NSC23766 (NSC) or vehicle, and then stimulated with 0.5 µ/ml convulxin under stirring at 37 °C. Aliquots were taken at the indicated time points and subsequently lysed with NP-40 detergent. Proteins were separated by reducing SDS-PAGE (10 %), blotted on a polyvinylidene difluoride membrane and probed with the phospho-specific cofilin (Ser3) antibody. Staining of the non-phosphorylated protein (total-cofilin) served as loading control. The results shown are representative of three individual experiments.

in platelet degranulation and lamellipodium assembly. In inactive state cofilin is present in phosphorylated form, whereas it becomes active after dephosphorylation. To investigate if cofilin dephosphorylation is mediated similarly in human and mouse platelets, and if dephosphorylation was affected by NSC23766, phosphorylation studies were performed with washed platelets after stimulation with the GPVI agonist *convulxin* (CVX). Western blot analysis showed that the amount of phosphorylated cofilin increased over time after GPVI stimulation with CVX in vehicle-treated murine wild-type platelets as well as in knockout platelets (Figure 3-12). Interestingly, under treatment with NSC23766 the amount of phosphorylated cofilin was constant at all time points after platelet activation. These results suggest that Rac1 is not involved in activation of cofilin, but the observed abolished dephosphorylation is rather a consequence of Rac1-independent effects of NSC23766. Quantification of the western blots confirms the dynamic increase of p-cofilin in untreated WT and KO platelets, which was abolished after treatment with NSC23766 (Figure 3-13).

Cofilin is a small protein downstream of PAK1, which in turn is a major effector protein of Rac1. To investigate if Rac1 binding to PAK1 was affected by NSC23766 and EHT1864, phosphorylation studies were performed as described in Figure 3-12, using phospho-specific and total PAK1/2 antibodies. After stimulation with thrombin, *Rac1*$^{+/+}$ platelets displayed a transient phosphorylation of PAK1/2 at threonine 423/402 over a time frame of 120 s. PAK1/2 phosphorylation in *Rac1*$^{-/-}$ platelets followed similar transient kinetics, but the amount of phosphorylated proteins was less pronounced (Figure 3-14). Application of NSC23766 remarkably impaired PAK1/2 phosphorylation in *Rac1*$^{+/+}$ and interestingly, further impaired PAK1/2

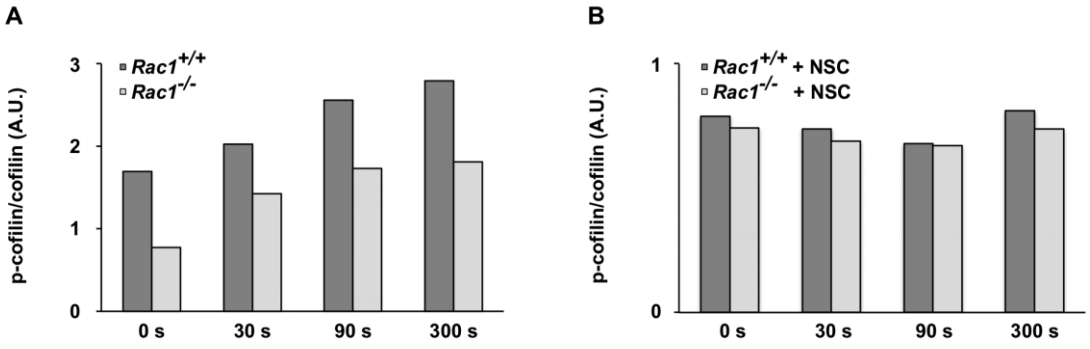

Figure 3-13: Quantification of p-cofilin after convulxin stimulation. (A) *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ exhibited slowly increasing levels of p-cofilin (inactivation) after initial convulxin stimulation. Platelets treated with NSC23766 do not exhibit dynamic change of p-cofilin levels, independently of presence or absence of Rac1 (B). The results shown are representative of three individual experiments. A.U., arbitrary units

phosphorylation in *Rac1*$^{-/-}$ platelets as compared with vehicle-treated *Rac1*$^{-/-}$ platelets (Figure 3-14). Application of EHT1864 completely abolished PAK1/2 phosphorylation in both *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ platelets.

All together, these results strongly suggest that NSC23766 and EHT1864 alter phosphorylation of the Rac1 effector molecules PAK1 and PAK2, independently of Rac1.

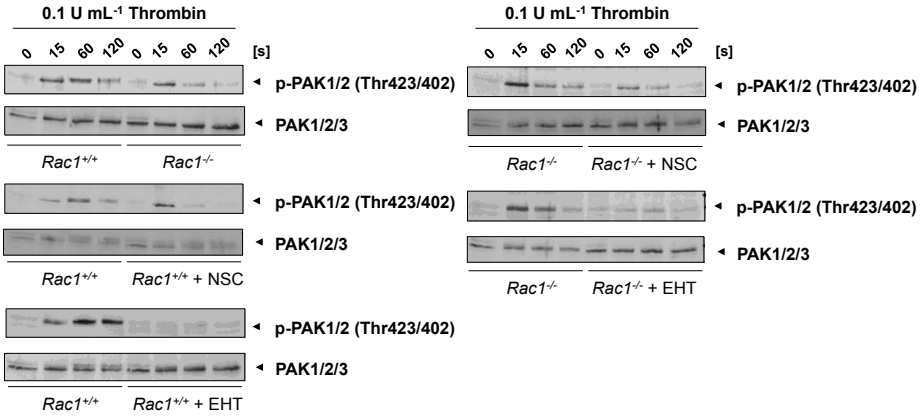

Figure 3-14: Rac1-independent inhibition of p21-activated kinase (PAK) 1/PAK2 activation. Washed platelets (7 x 10^5/µl) from *Rac1*$^{+/+}$ and *Rac1*$^{-/-}$ mice were incubated with NSC23766 (NSC), EHT1864 (EHT) and or vehicle, and then stimulated with 0.1 U/ml thrombin under stirring at 37 °C. Aliquots were taken at the indicated time points, and subsequently lysed with NP-40 detergent. Proteins were separated by reducing SDS-PAGE (10 %), blotted on a polyvinylidene difluoride membrane, and stained with the phospho-specific PAK1/PAK2 (Thr423/Thr402) antibody (MW 61 – 67 and 68 – 78 kDa). Staining of the respective non-phosphorylated proteins (PAK1/2/3; MW 61 – 68 kDa) served as loading control. The results shown are representative of three individual experiments (Dütting *et al*., *J Thromb Haemost*, 2015).

4 Discussion

Atherothrombotic diseases are the leading cause of morbidity and mortality worldwide[4]. Therefore, research on prevention of cardiovascular diseases has been given high priority. Different preventive approaches have been established, including medical therapy with *acetylsalicylic acid* (ASA) and $P2Y_{12}$ receptor antagonists, mainly targeting platelet signaling in order to prevent platelet aggregation, as well as anticoagulants like the vitamin K antagonist *Warfarin* and the *New oral anticoagulants* (NOACs) which inhibit blood coagulation. Although arterial thrombus formation can be effectively reduced, all available therapeutic drugs bear the risk of severe bleeding. This makes it still necessary to find new therapeutic agents and approaches to treat atherothrombotic diseases.

At sites of vascular injuries, platelet activation and aggregation are indispensable for wound sealing and limitation of blood loss; however, inadequate platelet activation may lead to pathologic formation of thrombi. Acute and chronic vessel occlusion in the coronary and cerebral circulation system by thromboembolic events can result in myocardial infarction or ischemic stroke. Endogenous platelet activation is most commonly triggered by second messengers or exposed extracellular matrix components at injured vessel walls, serving as platelet receptor agonists. Those stimuli induce a massive reorganization of the platelet cytoskeleton, leading to aggregation, adhesion and shape change and cumulating in thrombus formation for wound sealing.

Key regulators of these processes are among others the Rho GTPases Rac1, Cdc42 and RhoA which are molecular switches that regulate essential cellular processes and have pivotal functions in the cardiovascular system[39]. These Rho GTPases contribute to physiologic hemostasis, but also control pathologic thrombus formation[31–34]. Modulation of Rho GTPase activity could represent a feasible pharmacological approach for antithrombotic therapy and has emerged as important subject of research. Besides the development of knockout mouse models, different inhibitors of RhoGTPases have been tested in a wide range of experimental settings. Small-molecule inhibitors targeting Rac1 have recently been tested *in vitro* as well as *in vivo* for a variety of disorders, including acute myeloid leukemia, proteinuric kidney disease and diabetes[73–75].

In platelets, Rac1 is one of the major RhoGTPases which is responsible for maintaining intact signaling upon platelet activation. Proteomic studies suggest that human platelets express two of the known Rac isoforms, Rac1 and Rac2[76–78]. For mouse platelets it was found that Rac2 does not play a role as it was not detectable by immunoblotting and furthermore, Rac2 knockout studies by McCarty *et al.*[30] revealed no phenotypic changes in mouse platelets. However, human hematopoietic cells exhibit higher Rac2 expression compared to mice, and the importance of Rac2 in human platelets might be different[79,80].

The two small molecules EHT1864 and NSC23766 have been proposed to inhibit the Rac1 with high specificity[38,52,59]. Notably, EHT1864 is an inhibitor of not only Rac1 but also other Rac isoforms. The application of the inhibitors is not restricted to platelets, and so far they have rather been used in *in vitro* cell models other than platelets[81,82]. NSC23766 has even been applied *in vivo* in mouse models through intraperitoneal injections, intrathecal catheters and implanted Alzet osmotic pumps[73,74,83,84]. Considering that murine platelets only express the Rac1 isoform[30], mouse platelets represent an ideal model to investigate the specificity of NSC23766 and EHT1864 on Rac1 on a functional level.

4.1 NSC23766 and EHT1864 severely affect GP exposure and platelet viability

The first part of the results demonstrated dose-dependent inhibitory effects of NSC23766 and EHT1864 on platelet function. This could be observed by reduced integrin activation and P-selectin exposure in wild-type platelets after GPCR and (hem)ITAM receptor stimulation (Figure 3-1). Adequate inhibition of platelet activation was only achieved at concentrations from 100 µM upwards for both Rac1 antagonists, which is in line with the data of Gao *et al.* who reported a NSC23766-dosis of 50 µM as IC_{50}-value[38]. However, for EHT1864 potent inhibition was reported at concentrations of 5 µM[59], but in platelets it has been only used in remarkably higher concentration of 150 µM[43]. The different efficacies of EHT1864 may be explained by the length of incubation times: given the fact that EHT1864 induces apoptosis at high concentrations, shorter incubation times (5 min) may ensure the highest vitality of platelets at cost of inhibition efficacy. For platelets, we assessed a concentration of 100 µM as the most suitable drug dose, at which Rac1 inhibition was most sufficient while exhibiting the least off-target effects.

In line with the proposed role of Rac1 in the ITAM pathway, NSC23766 and EHT1864 inhibited activation upon GPVI-stimulation significantly. Interestingly, NSC23766 and EHT1864 also affected GPCR-triggered platelet activation in wild-type platelets and, more importantly, similarly in Rac1-deficient platelets (Figure 3-3). Severe GPVI-signaling defects are well established for Rac1-deficient platelets, whereas it is controversially discussed if Rac1-deficiency affects GPCR-mediated platelet activation. Yet, the presented GPCR-signaling defect in Rac1-deficient platelets, induced by the inhibitors, implicate an at least partially Rac1-independent effect of NSC23766 and EHT1864.

It has been shown that Mx-Cre-mediated Rac1-deficiency has no effect on platelet surface receptor expression[33], and in our study this was shown to be similar for PF4-Cre-mediated Rac1-deficient platelets. Interestingly, application of NSC23766 significantly reduced the expression of the major surface GPs, including integrin αIIbβ3, GPVI and GPV in wild-type and knockout platelets (Table 3-1). This suggests that NSC23766 triggers Rac1-independent processes which diminish GP surface expression. It is not clear if decreased expression of membrane receptors could be attributed to reduced platelet reactivity upon treatment with NSC23766. The exact mechanisms of GP downregulation after treatment with NSC23766 remain unidentified and further experimental investigations would be needed for clarification. Importantly, these data should rather serve as a note of caution regarding the use of high NSC23766 concentrations in future.

Flow cytometry-based analysis revealed that EHT1864 significantly increased the amount of cell debris formation (Figure 3-2) and led to enhanced PS exposure. PS is well known to be an early indicator of platelet apoptosis as well as a signal to initiate blood clotting[61]. In collaboration with the laboratory of M. R. Ahmadian we could confirm that EHT1864 cause the decline of mitochondrial membrane depolarization[63] and thus platelet apoptosis. Tiede *et al*. reported in 2003 that the commercially used immunosuppressive drug azathioprine and its metabolites directly bind to Rac1 and block the nucleotide exchange, which induces a mitochondrial pathway of T cell apoptosis[85]. Comparably to the interaction of Rac1 and azathioprine, EHT1864 binds to Rac1 and disrupts the guanine nucleotide association. It is plausible that platelet apoptosis upon EHT1864 might follow similar mechanisms as upon azathioprine. This raises further questions if EHT1864 may also induce apoptosis in other cell

types like lymphocytes or erythrocytes by inhibition of Rac1, which would lead to severe side effects if applied *in vivo*.

Platelet apoptosis could not be observed after treatment with NSC23766. In contrast to EHT1864, NSC23766 leaves Rac1 in inactive GDP-bound state, which may not induce mitochondrial-mediated apoptosis. The molecular mechanism of apoptosis upon EHT1864 remains unclear. Nonetheless, high inhibitor concentrations should be used carefully in platelets. To date, no *in vivo* studies have been published using EHT1864 and on basis of the presented results it is not advisable to apply EHT1864 as *in vivo* inhibitor. The observed platelet apoptosis might significantly enhance bleeding risk and prolong bleeding time in laboratory animals.

4.2 Significant Rac1-independent effects in platelet inside-out signaling upon NSC23766 and EHT1864.

It is well known that Rac1-deficiency in platelets leads to profound defects in (hem)ITAM-signaling, whereas the role of Rac1 in GPCR signaling is discussed controversially[32,42]. We observed a discrete signaling defect after thrombin-induced GPCR stimulation in PF4-Cre Rac1-deficient platelets. Similarly, GPCR-signaling was diminished in wild-type platelets after treatment with the Rac1 antagonists. Since EHT1864 inhibits activity of Rac isoforms other than Rac1, it is tempting to speculate that mouse platelets may indeed express Rac2, since proteomic analyses revealed that Rac2 is highly expressed on the mRNA level in human platelets[77]. In fact, Akbar *et al*.[86] discussed adhesion- and aggregation defects in mouse platelets isolated from Rac2 gene targeted knockout mice. However, data of these findings have never been published[87]. Since McCarty *et al.* clearly disproved presence or upregulation of Rac2 in wild-type and Rac1 knockout platelets by immunoblots[30], a role of Rac2 in mouse platelets is highly unlikely. Therefore, it is rather implausible, that the observed GPCR signaling defect was induced by Rac2-inhibition of NSC23766 and EHT1864.

The data from Pleines *et al.*[32] and Akbar *et al.*[42] were obtained from platelets isolated from Mx-Cre mice. These mice lack Rac1 in all hematopoietic cell lines, whereas the PF4-Cre-mediated deletion is restricted to MKs and platelets. Rac1 deletion in *Mx-Cre* mice is induced in adult animals, whereas PF4-Cre mice do not express Rac1 in platelets since embryonic age. The observed discrete discrepancies in GPCR signaling make room for further speculations about the role of Rac1 downstream of GPCRs in mouse platelets and the effects of different deletion strategies.

For human platelets it was shown that stimulation of GPCRs leads to Rac1-mediated activation of PAK2 and PI3K[43], suggesting that inhibition of Rac1 affects downstream signal transduction. It is tempting to speculate that this can be transferred to murine platelets, explaining the observed GPCR signaling defect in Rac1-deficient platelets and after treatment with the inhibitors. However, the inhibitors further enhanced the GPCR signaling defect in *Rac1*$^{-/-}$ platelets (Figure 3-3). This strongly suggests that both Rac1 antagonists target other molecules within GPCR signaling pathways apart from Rac1.

At site of vessel injuries, the ligand occupation of integrin αIIbβ3 triggers integrin outside-in signaling and leads to cytoskeletal reorganization and platelet spreading[13], which is essential for physiological wound closure. Rac1-deficient platelets expose severe defects in lamellipodia-formation[30], while filopodia-formation remains intact. NSC23766- and EHT1864-mediated Rac1 inhibition only had minor effects on outside-in signaling in wild-type platelets (Figure 3-7). Platelet spreading was only slightly decreased upon 100 µM Rac1-inhibitor and platelets retained the ability to form lamellipodia. Since Rac1 is indispensable for lamellipodia formation, these data demonstrate lacking efficacy of NSC23766 and EHT1864. Besides, both inhibitors revealed Rac1-independent effects, as spreading of Rac1-deficient platelets was diminished, too.

Integrin αIIbβ3 outside-in signaling also regulates clot retraction[67], an essential process for wound healing. Previous studies reported defective clot retraction upon platelet-treatment with NSC23766 or EHT1864, suggesting an important role of Rac1 in this process[66,68]. Our data strongly suggest that Rac1 is dispensable for clot retraction and furthermore indicate that defective clot retraction from previous data was a result from Rac1-independet inhibition by NSC23766 and EHT1864[66,68]. Stefanini *et al*.[66] showed that clot retraction was remarkably reduced in EHT1864-treated wild-type platelets as compared to controls. However, the pronounced defect in clot retraction might rather be explained by the high inhibitor concentration of 150 µM which induces platelet apoptosis as proved by this thesis. In line with this, treatment with 100 µM EHT1864 affected clot retraction to a lesser extent, similar to the observed dose-dependent induction of apoptosis[63].

Clot retraction was not affected by treatment with NSC23766 which stands in sharp contrast to Flevaris *et al*.[68], who showed defective clot retraction in NSC23766-

treated wild-type platelets with inhibitor concentrations of only 20 µM. Notably, we did not observe sufficient inhibition of platelet activation at concentrations lower than 50 µM (Figure 3-1). By contrast, Flevaris *et al*. incubated platelets with inhibitors significantly longer prior to clot retraction (30 min) than we did (5 min), which might explain a higher efficacy of the compound. However, for *in vitro* clot retraction platelets have to retain highest possible viability. Prolonged *in vitro* incubation times may affect platelet viability and result in defective clot retraction.

Interestingly, within the same study Flevaris *et al*.[68] showed defective clot retraction in platelets isolated from mice with *Mx-Cre*-mediated gene deletion, which stands in contrast to our data obtained from *PF4-Cre* mouse lines. It is likely that the outcome of the performed experiments at least in part seems to be influenced by the used *Cre*-mouse line (Mx-Cre vs. PF4), but further experimental evidence is needed to clarify the underlying cause for these different phenotypes.

It was shown that platelet spreading on vWF-coated surfaces under conditions of αIIbβ3 blockade is diminished in Rac1-deficient platelets isolated from *Mx-Cre* mice[71]. On vWF-coated surfaces, the membrane receptor GPIb induces platelet shape change that is limited to cell body contraction and formation of long thin filopodia. In 2012 Delaney *et al.* identified Rac1 as a downstream effector of Lyn, thereby playing an important role in GPIb-IX-mediated platelet activation. This was shown by use of PF4-Cre-mediated *Rac1* deletion, as well as in NSC23766-treated human platelets[72]. Additionally, we could show that both inhibitors decrease platelet spreading on vWF-coated surfaces; however, again both inhibitors further diminished filopodia formation in Rac1-deficient platelets as compared with vehicle-treatment, revealing Rac1-independent off-target effects of NSC23766 and EHT1864.

4.3 NSC23766 and EHT1864 affect PAK1/PAK2 activity

Although being characterized as specific inhibitor for Rac1 and its isoforms[38], Levay *et al.* recently demonstrated that NSC23766 is also a non-selective, competitive antagonist of muscarinic acetylcholine receptors[88]. Binding modeling revealed that NSC23766 docks well to the orthosteric binding pocket of M2 and M3 muscarinic acetylcholine receptors. With this, a specific off-target molecule of NSC23766 was identified for the first time. Until now, no specific off-targets were reported for EHT1864. Importantly, the data by Levay *et al.* question the specificity of NSC23766 as Rac1 inhibitor. In fact, data regarding Rac1 function, which have been obtained

from experiments using inhibitor-treated wild-type platelets but without confirmation in Rac1-deficient platelets, have to be scrutinized thoroughly.

Rho GTPase GEFs show high homologies in their molecular structure. It seems possible that NSC23766 might fit into GEF binding grooves in the molecular structure of Cdc42 and RhoA, and target these proteins in absence of all Rac isoforms. It has been demonstrated multiple times that nucleotide exchange of Cdc42 and RhoA is not affected by NSC23766 and EHT1864[43,82] and G-LISA assays confirmed this in *Rac1*$^{-/-}$ platelets (Figure 3-10). Yet, it might be possible that NSC23766 and EHT1864 interfere with signal transduction downstream of these Rho GTPases, independently of intact nucleotide exchange. However, off target effects in platelets with double-deficiency for either Rac1 and Cdc42, or for Rac1 and RhoA with wild-type platelets were similar to effects seen in platelets from Rac1 single knockout mice (Figure 3-11).

It was found by Pandey *et al*. that NSC23766 prevents dephosphorylation of cofilin in human platelets, concluding that Rac1 is an important mediator of cofilin activation[43]. Our data revealed that vehicle-treated Rac1-deficient mouse platelets exhibit intact cofilin dephosphorylation/phosphorylation; however, the transient dephosphorylation was abolished upon treatment with NSC23766. This unravels a distinct off-target effect of NSC23766 and puts the findings of Pandey *et al.* into question. Inhibition of cofilin dephosphorylation by NSC23766 might also explain the observed off-target effects in αIIbβ3-mediated outside-in signaling in mouse platelets. Yet, we only investigated mouse platelets, and the role of Rac1 regarding cofilin dephosphorylation might still be different in human platelets.

Cofilin is a downstream effector of LIMK1, which is activated by ROCK and more importantly also by PAK1. PAKs are direct downstream effectors of Rac1 and Cdc42 in almost all cell types. Rac1-GTP binds to PAK1/PAK2, inducing auto-phosphorylation of PAK1 (Thr423) and PAK2 (Thr402) which is necessary for their activation and for subsequent signal propagation[89]. The performed experiments confirmed significantly impaired thrombin-induced activation of PAK1/PAK2 in Rac1-deficient platelets (Figure 3-14). Furthermore, PAK1/PAK2 activity was completely abolished in NSC23766 and EHT1864-treated wild-type platelets and Rac1-deficient platelets, likewise. Data from a cell-free PAK activity assay later confirmed Rac1-independent affection of PAK1/PAK2 activity by NSC23766 and EHT1864[63]. This

influence of the two compounds may also explain the observed Rac1-independent defects in platelet activation upon GPCR stimulation. Considering Levay *et al.* who demonstrated that NSC23766 also is a non-selective, competitive agonist of muscarinic acetylcholine receptors and binds directly into the M2 and M3 binding pocket[88]. It is tempting to speculate that NSC23766 might also bind directly to PAK1 and PAK2, thereby inhibiting auto-phosphorylation, which causes the non-specific effects.

4.4 Concluding remarks

The findings summarized in this thesis shed new light on the specificity and efficacy of the two Rac(1) inhibitors NSC23766 and EHT1864. The newly found interactions with PAK1/PAK2 questions further use of the inhibitors for research on Rac1. The use of knockout mouse models should be preferred over the use of inhibitors.

The observed effects of NSC23766 and EHT1864 confine the potential of becoming novel therapeutic agents for antithrombotic therapy. Rac1 is ubiquitously present which may imply problematic effects not only on other hematopoietic cell lines. It is necessary to investigate if EHT1864-mediated apoptosis can be observed in other Rac1-bearing cell-types, such as $CD4^+$ T-cells. Furthermore, *in vivo* application of EHT1864 has to be taken with utmost care as apoptosis may similarly occur under *in vivo* conditions, leading to severe thrombocytopenia or immunosuppression.

Rac1-deficient mice exhibit prolonged bleeding times in *in vivo* tail bleeding experiments. Although data about *in vivo* effects of NSC23766 by intraperitoneal injection has been published, nothing is known about the effects on platelet function. Further investigation would be necessary to determine if the Rac1-inhibitors lead to enhanced bleeding risk when applied *in vivo*.

In summary, the small molecule inhibitors NSC23766 and EHT1864 have to be used carefully when used in *in vitro* platelet experiments. It is questionable if these compounds have the potential for novel therapeutic agents and further detailed studies would be indispensable to characterize the systemic effect of NSC23766 and EHT1864 *in vivo*.

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5 Appendix

5.1 Abbreviations

AC	adenylyl cyclase
ADP	adenosine diphosphate
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
BSA	bovine serum albumin
Cdc42	cell division control protein 42
CLEC-2	C-type lectin-like receptor type 2
COX	cyclooxygenase
CRP	collagen-related peptide
CVX	convulxin
DAG	diacylglycerol
DIC	differential interfering contrast
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol tetraacetate
FcR	Fc receptors
Fg	fibrinogen
FITC	fluorescein isothiocyanate
FSC	forward scatter
g	gravity
GAP	GTPase-activating proteins
GDI	guanine nucleotide-dissociation inhibitors
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GP	glycoprotein
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
GTPase	guanosine 5'-triphosphatase
h	hour
Ig	immunoglobulin
IP_3	inositol-3,4,5-triphosphate
ITAM	immunoreceptor tyrosine-based activation motif
kDa	kilodalton
LAT	linker for activation of T cells
LIMK	LIM domain kinase
MFI	mean fluorescence intensity
min	minute
MK	megakaryocyte(s)
NOAC	New oral anticoagulant(s)
PAK	p21 activated kinase
PAR	protease-activated receptor(s)

PBS	Phosphate-buffered saline
PE	phycoerythrin
PF4	platelet factor 4
PFA	Paraformaldehyde
PGI_2	Prostacyclin
PI3K	phosphoinositol-3-kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-trisphosphate
PKC	protein kinase C
PLC	phospholipase C
Poly I:C	polyinosinic-polycytidylic acid
prp	platelet-rich plasma
PS	phosphatidylserine
PVDF	polyvinylidene difluoride
Rac1	Ras-related C3 botulinum toxin substrate 1
RC	rhodocytin
RhoA	Ras homolog gene family, member A
RT	room temperature
ROCK	Rho-associated protein kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFK	Src family kinase
SSC	side scatter
SOCE	store-operated Ca^{2+} entry
Syk	spleen tyrosine kinase
TBS	Tris-buffered saline
TF	tissue factor
TRCP	transient receptor potential channel
TxA_2	thromboxane A_2
vWF	von Willebrand Factor

5.2 Acknowledgements

5.3 Publications

5.3.1 Original articles

Dütting S, **Heidenreich J**, Cherpokova D, Amin E, Zhang SC, Ahmadian MR, Brakebusch C, Nieswandt B. Critical off-target effects of the widely used Rac1 inhibitors NSC23766 and EHT1864 in mouse platelets. *J Thromb Haemost*. 2015; 13(5):827-38.

5.3.2 Posters

9th International Symposium of the Graduate School of Life Science, 2014, Wurzburg:

Rac1-independent effects of widely used inhibitors NSC23766 and EHT1864 in platelet activation.

(Germany)

5.4 Curriculum vitae

5.5 Affidavit

I hereby confirm that my thesis entitled, “Characterization of the widely used Rac1-inhibitors NSC23766 and EHT1864 in mouse platelets”, is the result of my own work. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

Würzburg, December 2017_____

Julius Frederik Heidenreich

5.6 Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation „Untersuchungen der kommerziellen Rac1-Inhibitoren NSC23766 und EHT1864 in murinen Thrombozyten“ eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, Dezember 2017_____

Julius Frederik Heidenreich