EXP3179 Inhibits Collagen-Dependent Platelet Activation via Glycoprotein Receptor-VI Independent of AT₁-Receptor Antagonism
Potential Impact on Atherothrombosis

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Objective—Thrombus formation after atherosclerotic plaque rupture critically involves the platelet collagen receptor glycoprotein (GP) VI. We investigated the impact of EXP3179, an active metabolite of the angiotensin II type 1 (AT₁)-receptor antagonist Losartan (LOS) on GPVI-dependent platelet activation.

Methods and Results—EXP3179 and LOS but not EXP3174—the major AT₁-receptor blocking metabolite of LOS—dose-dependently inhibited collagen-I (P < 0.01) and GPVI-dependent platelet aggregation (P < 0.01) analyzed by optical aggregometry. Platelet activation was further determined by flow cytometry measuring the expression of platelet PAC-1, an epitope of the activated fibrinogen-receptor complex. EXP3179 and LOS inhibited collagen-I (P < 0.01) and GPVI-dependent PAC-1 expression (P < 0.01). EXP3179 and LOS but not EXP3174 decreased the adhesion of GPVI-receptor expressing Chinese hamster ovarian cells on collagen-I under arterial shear conditions determined by flow chamber analysis (P < 0.01 and P < 0.05). EXP3179 also reduced human atherosclerotic plaque material-induced platelet aggregation (P < 0.01) in vitro and murine platelet adhesion after acute vessel injury in vivo as determined by intravital microscopy (P < 0.01).

Conclusion—EXP3179 acts as a specific inhibitor of the platelet collagen receptor GPVI independent of AT₁-receptor antagonism. Further investigations may clarify its individual potential as a novel pharmacological approach to specifically inhibit atherothrombotic events by GPVI-receptor blockade. (Arterioscler Thromb Vasc Biol. 2007; 27:1-2.)

Key Words: EXP3179 | platelets | collagen | GPVI-receptor | atherothrombosis

Occulsive arterial thrombosis after atherosclerotic plaque rupture represents the major pathophysiological mechanism underlying acute coronary syndromes (ACS) or cerebrovascular events, ie, stroke.¹ Plaque rupture injures the integrity of the vascular wall and leads to exposure of highly procoagulatory extracellular matrix (ECM) components.²,³ These include collagen-I, a major element of the structurally altered ECM in atherosclerotic vessels.⁴-⁶ The platelet collagen receptor glycoprotein (GP)VI seems to be crucial for thrombus formation at sites of murine arterial vascular injury and in response to platelet contact with human atherosclerotic plaque material.⁷,⁸,⁹ Subsequently, platelet GPVI-receptor surface expression was found to be elevated in patients with ACS.¹⁰ Thus, blockade of GPVI-receptor activation may represent a novel pharmacological target to inhibit thrombotic events after atherosclerotic plaque rupture.

EXP3179 was originally identified as an active metabolite of the angiotensin II type 1 (AT₁)-receptor antagonist Losartan (LOS), which is produced during the hepatic metabolism of LOS by the cytochrome-P450 pathway.¹¹ We recently reported that EXP3179 is detectable in patients after LOS administration and inhibits platelet activation, even though the underlying mechanisms remained unclear.¹² Current observations from other groups revealed that EXP3179 activates the endothelial nitric oxide synthase (eNOS) and acts as a peroxisome proliferator-activated receptor (PPAR)-gamma
agonist. So far, effects of EXP3179 have only been investigated in dependence on LOS treatment. In contrast, we here postulated that EXP3179 might possess an individual pharmacological potential independent of LOS application and the AT1-receptor. Therefore, we investigated the effects of EXP3179, LOS, and EXP3174—the main metabolite of LOS and specific AT1-receptor antagonist—on collagen-I and GPVI-receptor–dependent platelet activation, adhesion, and aggregation in vitro and the impact of EXP3179 on acute platelet adhesion to the injured arterial wall in vivo.

Materials and Methods

Synthesis of EXP3179
The H- and 13CNMR spectra were recorded on a Bruker AC 300 spectrometer at 300 (75). Chemical shifts are reported as ∆ values (ppm) downfield from Me4Si. Mass spectrometry was performed on a Bruker-Franzen Esquire LC mass spectrometer. Flash column chromatography was carried out using Merck silica gel 60 (40 to 63 and 15 to 40 μm) and 60G (5 to 40 μm). Thin-layer chromatography (TLC) was carried out using aluminum sheets precoated with silica gel 60 F254 (0.2 mm; Merck). Chromatographic spots were visualized by UV and/or spraying with an acidic, ethanolic solution of p-anisaldehyde or an ethanolic solution of ninhydrin followed by heating. All commercial chemicals were used without further purification. EXP3179 was synthesized by a modified protocol providing higher yields than previously reported methods. Losartan (0.422 g, 1 mmol) was oxidized with IBX (2-iodoxybenzoic acid, 0.321 g, 0.321 mmol) in DMSO (5 mL) at room temperature for 6 hours. CHCl3 (30 mL) was added and the solution was washed with water (3×30 mL), NaHCO3 solution (3×30 mL, sat.), and brine (1×30 mL). The solvent was removed after drying (Na2SO4) in vacuo to yield EXP3179 (410 mg, yield 98%, molecular structure of EXP3179, Figure 1A).

Synthesis of EXP3174 via EXP3179
A mixture of 0.147 g (3.3 mmol) of EXP3179 and 0.287 g (3.3 mmol) of activated manganese dioxide in 2 mL of H2O was refluxed for 78 hours. The reaction was monitored by TLC (CHCl3/MeOH/CH3COOH 95/5/0.5). Excess of MnO2 was filtered, the solvent was removed under reduced pressure and the residue was purified by acid base workup to yield EXP3174 (200 mg, 56%).

EXP3179, Losartan, and EXP3174 Stock Solutions
EXP3179 was dissolved in 0.05% DMSO (Sigma-Aldrich), 9.95% Tris-HCl and diluted in phosphate buffered saline (PBS, pH 7.4, free of Ca2+ and Mg2+). Excess of MnO2 was filtered, the solvent was removed under reduced pressure and the residue was purified by acid base workup to yield EXP3179 (200 mg, 56%).

Blood Samples
Human peripheral venous blood samples were taken with a loose tourniquet to avoid artifacts through a short venous catheter inserted into the forearm of healthy volunteer donors, who had not taken any medication known to interfere with platelet activation for at least 10 days.

Aggregometric Analysis
Platelet aggregation was evaluated by optical aggregometry as described previously. After adjustment to a total platelet count of 2×108/mL, platelet-rich plasma (PRP) was incubated with varying doses of EXP3179, LOS, EXP3174 or the corresponding solvents for 15 minutes. Collagen (type-I) (Proteo&Go, Germany) or 4C9, a monoclonal GPVI-receptor stimulating antibody were added in varying concentrations to induce platelet activation. As reported previously, 4C9 detects the GPVI-receptor on the platelet surface. Adenosin-5′–diphosphate (ADP, 5 μmol/L; Proteo&Go) or thrombin-receptor-activating peptide (TRAP) (25 μmol/L; Sigma-Aldrich) were additionally used to induce platelet activation. To evaluate the impact of EXP3179 on atherosclerotic plaque material-induced platelet aggregation, we followed a protocol recently published by Penz et al. Patient consent was obtained as approved by the Institutional Ethics Committee. Maximal aggregation at 5 minutes was used as measurement of aggregation.

Flow Cytometry
Evaluation of the surface expression of platelet membrane glycoproteins was performed by immunolabeling followed by flow cytometric analysis as described previously. In brief, PRP (adjusted to 2×108 platelets/mL) was incubated with varying doses of EXP3179, LOS, EXP3174, or the corresponding solvents for 30 minutes. After incubation with varying dosages of 4G9 or collagen-I and PAC-1 antibody, an epoxide of the activated fibrinogen receptor complex (anti–PAC-1 fluorescein isothiocyanate [FITC], Becton Dickinson) for 30 minutes. After staining, the cells were fixed with 0.5% paraformaldehyde and stored at 4°C until fluorescence activated-cells sorting (FACS) was performed with a FACS-Calibur flow cytometer (Becton-Dickinson). 10,000 events were analyzed. Specific antibody binding was expressed as mean intensity of immunofluorescence and was used as a quantitative measurement of platelet protein surface expression.

Flow Chamber
Experiments were basically performed as previously described. Glass cover slips for usage in a flow chamber were coated with collagen type I. Human whole blood from healthy volunteer donors, who had not taken any medication for at least 10 days before the experiment, was collected into syringes containing 0.5% vol% heparin. To analyze and quantify platelet adhesion to collagen under arterial flow conditions, platelets were labeled in whole blood by incubation with the fluorescent dye rhodamine-6G (final concentration 0.2 g/L) for 15 minutes at 37°C. Leukocytes could be readily distinguished from platelets by their larger size and nuclear morphology; red cells were not visualized by rhodamine-6G. Perfusion was performed for 5 minutes at 1000/s followed by a 5-minute perfusion with HEPES-modified Tyrode solution at a shear rate of 1000/s using a pulse-free pump. The flow chamber was mounted on an inverted fluorescence microscope (Axiovert, Zeiss) and fluorescent images were recorded from 5 to 8 different microscopic fields (20× objectives) using a digital photo camera (AxioCam, Zeiss). The number of firmly adherent platelets was counted offline from the recorded fluorescent using a computer-assisted image analysis program (Cap Image 7.1; Zeintl).

To investigate the impact of EXP3179, LOS, and EXP3174 on GPVI-receptor–mediated cell adhesion on collagen-I under shear conditions, we stably transfected CHO cells with the human GPVI-receptor using the Flp-In system (Invitrogen) as described elsewhere. Non-transfected Flp-In CHO cells served as controls. CHO-GPVI and control CHO cells were cultivated using HAM F12 containing 10% FCS ± 1% penicillin/streptomycin. For culturing the CHO-GPVI cell line 400 μg/mL hygromycin were added. Surface expression of the human GPVI-receptor by CHO cells was tested by flow cytometry using the anti-GPVI monoclonal antibody 5C4 (please see supplemental Figure III, available online at http://atvb.ahajournals.org). Both cell lines were perfused over collagen-I (20 μg/mL) or bovine serum albumin (BSA, 3%) coated glass coverslips in a cell concentration of 500,000/mL at a constant shear rate of 1000 sec−1 for 5 minutes. Experiments were recorded in real time and evaluated off-line using Cap Image software (Cap Image 7.1; Zeintl).

Intravital Fluorescence Microscopy
For intravital fluorescence microscopy (IVM) of the injured carotid artery, 12-week-old C57BL6/J mice (Charles River; Sulzfeld, Germany) were anesthetized by intraperitoneal injection of Midazolam (5 mg/kg bodyweight), Medetomidine (0.5 mg/kg bodyweight), and Fentanyl (0.05 mg/kg bodyweight). Murine platelets were isolated from whole blood samples and labeled with 5-(and -6)-carboxyfluorescein diacetate, succimidyl ester (DCF). The final platelet concentration was 2×108/500 μL PRP. After preincubation of murine platelets with either EXP3179 or the diluent (15 minutes), samples were administered via a jugular catheter. Adhesion of fluorescent platelets was analyzed by in situ video microscopy before and after.
carotid injury caused by ligation of the common carotid artery for 5 minutes as described previously. Adherent platelets were measured using a computer-assisted program and are given per mm² (Capimage, Zeintl, Germany). All experiments were approved by the Institutional Animal Care and Use committee.

Statistics
Data are given as mean±SD of at least 3 independent experiments per group. The 2-tailed unpaired t test was performed to compare differences between two groups. A probability value of <0.05 was considered as statistical significant.

Results
EXP3179 and LOS Dose-Dependently Inhibit Collagen-I Induced Aggregation and Activation of Human Platelets
After incubation of PRP with EXP3179, LOS, EXP3174, or solvent platelet aggregation was induced by stimulation with collagen-I and analyzed by optical aggregometry. EXP3179 and LOS dose-dependently inhibited collagen-I-induced platelet aggregation reaching significant effects at a concen-
tration of 500 μmol/L after stimulation of PRP with 1 and 2 μg/mL collagen-I (EXP3179 10±6.2%, LOS 20±9%, each versus PRP 63±9%, P<0.01, n=3 to 6, Figure 1B). In contrast, EXP3174 did not influence collagen-I dependent platelet aggregation. We did not find any impact of EXP3179 on ADP or TRAP-dependent platelet aggregation (data not shown).

Platelet activation leads to a rapid conformational change of the GPⅡb-Ⅲa receptor complex enabling soluble plasmatic fibrinogen binding, which is considered as a major step toward platelet aggregation. The impact of EXP3179 on GPⅡb-Ⅲa activation was evaluated by flow cytometry using the conformation-dependent antibody PAC-1, an epitope of the activated fibrinogen receptor complex. Platelets were preincubated with EXP3179, LOS, EXP3174, or solvent followed by incubation with collagen-I (for dose-response experiments please see supplemental Figure I). EXP3179, LOS and—to a lesser extent EXP3174—dose-dependently reduced the expression of PAC-1 reaching a maximum effect at a concentration of 500 μmol/L after stimulation of PRP with 5 μg/mL collagen-I (EXP3179 11.5±2, P<0.01, LOS 12.9±1.8, P<0.01, EXP3174 33.75±9.9, P<0.05, each versus stimulated PRP 65.82±19.3, n=4 to 7, Figure 1C). There was no difference between the stimulation of solvent+PRP or PRP alone with collagen-I (supplemental Figure I). EXP3179 did not affect TRAP- or ADP-induced PAC-1 expression (data not shown).

EXP3179 Reduces Human Platelet Adhesion on Collagen-I Under Shear Conditions
Platelet adhesion under dynamic shear conditions—as present in vivo—can be simulated by using a flow chamber. As reported by other groups, LOS and EXP3174 may influence collagen-dependent adhesion of human platelets under flow. To evaluate the impact of EXP3179 on this parameter, whole blood samples were labeled with rhodamin-6G and incubated with solvent or EXP3179 for 15 minutes before perfusion. Platelet adhesion was investigated under constant perfusion over collagen-I coated cover slips. EXP3179 significantly inhibited platelet adhesion evaluated under high shear rate (1000sec⁻¹) (44.7±14.4% versus 100%; n=5; Figure 1D) in comparison to solvent. We also performed this experiment with isolated platelets and found similar results (data not shown).

EXP3179 and LOS Inhibit GPⅥ-Receptor–Dependent Human Platelet Activation and Aggregation
To further evaluate the potential mechanisms underlying the antiaggregatory effects of EXP3179 following collagen stimulation, we analyzed the impact of EXP3179, LOS, and EXP3174 on the activation of the platelet collagen receptor GPⅥ. Therefore, human PRP was preincubated with EXP3179, LOS, or EXP3174 and stimulated with the selective GPⅥ-receptor activating antibody 4C9. EXP3179 and LOS dose-dependently inhibited GPⅥ-dependent platelet aggregation reaching a maximum effect at a concentration of 500 μmol/L after stimulation of PRP with 0.1 μg/mL 4C9. EXP3174 did not have any detectable influence on this parameter (EXP3179 18.8±4.9%; LOS 20±9.6%, each versus PRP 70.3±1%, each P<0.01, Figure 2A). Furthermore, flow cytometry experiments revealed that the 4C9-dependent platelet activation represented by PAC-1 expression was substantially reduced in the presence of EXP3179 and LOS reaching a maximum effect at a concentration of 500 μmol/L after stimulation of PRP with 0.1 μg/mL 4C9 (for dose-response experiments, please see supplemental Figure II; EXP3179 25.2±5.8, LOS 33.2±9.7, each versus stimulated PRP 508.4±18.9, each P<0.01, Figure 2B). EXP3174 did not significantly influence 4C9-dependent platelet activation. There was no difference between the stimulation of solvent+PRP or PRP alone with 4C9 (supplemental Figure II).

EXP3179 and LOS Inhibit GPⅥ-Receptor Dependent Cell Adhesion on Collagen-I Under Shear Conditions
To further specify the influence of EXP3179 on GPⅥ-receptor dependent cell adhesion under shear conditions, GPⅥ receptor–expressing CHO cells were used as previously described. EXP3179 and LOS but not EXP3174 (500 μmol/L) significantly inhibited the adhesion of CHO-GPⅥ cells on collagen-I–coated cover slips in comparison to solvent or untreated CHO-GPⅥ cells (EXP3179 14.3±9 versus 40.9±8.7 CHO-GPⅥ cells/mm², P<0.01, LOS 30.1±9.7 versus 40.9±8.7 CHO-GPⅥ cells/mm², P<0.05, n=3, Figure 3). We did not find any impact on CHO-GPⅥ cell adhesion on collagen-I after incubation with the different solvents or another AT1-receptor antagonist, Irbesartan (supplemental Figures IV and V). Neither EXP3179 nor LOS influenced the adhesion of control CHO cells (supplemental Figure VI).

EXP3179 Decreases Human Platelet Aggregation After Stimulation With Human Atherosclerotic Plaque Material
Thrombus formation at sites of atherosclerotic lesions seems to be critically influenced by collagen-induced platelet adhesion via GPⅥ-receptor activation. Therefore, we investigated the impact of EXP3179 on platelet aggregation in response to components of human carotid atherosclerotic plaques by aggregometry. After incubation with solvent or EXP3179, PRP was stimulated with homogenized carotid atherosclerotic plaque material from patients who had undergone carotid thrombendarterectomy. EXP3179 inhibited platelet aggregation after stimulation with human plaque homogenates (18.7±19.8% versus 56.7±16.7%, P<0.01; n=4; Figure 4) compared with solvent.

EXP 3179 Reduces Murine Platelet Aggregation In Vitro and Platelet Adhesion In Vivo
Stable murine platelet adhesion and aggregation at sites of acute vessel injury in vivo are substantially influenced by GPⅥ-receptor availability. Therefore, we evaluated the impact of EXP3179 on murine platelet aggregation and adhesion. To investigate potential species-specific differences of EXP3179, murine platelet aggregation was first investigated in vitro. As shown for human platelets, EXP3179 significantly inhibited murine platelet aggregation after stimulation with collagen-I in comparison to PBS (data not
shown) or solvent administration (25.3±24.8% versus 79.5±7.6%; *P<0.01; n=8 animals). PRP was pooled for 3 experiments; Figure 5A). To evaluate the influence of EXP3179 on murine platelet function in vivo, a mouse model of carotid injury was used and platelet adhesion at the site of injury was visualized by intravital fluorescence microscopy, as described previously.3 Murine DCF-labeled platelets were preincubated with EXP3179 or solvent and administered via a venous catheter. Carotid injury was induced by ligature of the common carotid artery as described elsewhere.3 Preincubation of murine platelets with EXP3179 significantly reduced platelet adhesion to the site of injury in comparison to solvent. (Figure 5B, 1061.5±298.1 versus 2047.0±692.2 adherent platelets/mm2; *P<0.01 vs CHO-GPVI adhesion on collagen-I; #P<0.05 vs CHO-GPVI adhesion on collagen-I; n=3 to 7).

Discussion

The major finding of our study is that EXP3179 acts independent of AT1-receptor antagonism as an individual and selective inhibitor of GPVI-receptor dependent platelet activation and aggregation in vitro and in vivo.

Acute vessel injury by atherosclerotic plaque rupture exposes procoagulatory elements of the subendothelial ECM to circulating platelets, which are instantly activated. Subsequently, occlusive arterial thrombosis may occur and result in ACS or stroke.1 In this regard, collagen-I is one of the major procoagulant ECM components in atherosclerotic vessels, which plays a critical role for stable platelet adhesion at the
The major platelet collagen receptor is considered to be GPVI as suggested by recent experimental and clinical evidence showing the importance of GPVI-receptor activation in collagen-dependent platelet adhesion and thrombus formation, both in vitro and in vivo. Interestingly, activation of GPVI may especially be critical for thrombotic events after atherosclerotic plaque rupture. Thus, inhibition of GPVI-receptor activation may represent a novel pharmacological target in the search of more selective and specific antithrombotic agents for the prevention and/or treatment of acute occlusive arterial thrombosis, eg, myocardial infarction or stroke. Blockade of the renin–angiotensin system (RAS) by AT1-receptor antagonists such as LOS effectively reduces the incidence of cardiovascular events as demonstrated by large scale clinical trials. Based on recent observations which revealed various potentially vasoprotective effects of EXP3179, we hypothesized that EXP3179 might possess an individual pharmacological potency, which may not only contribute to the beneficial effects of LOS but also qualitatively reduce the incidence of cardiovascular events as demonstrated by large scale clinical trials. This impact involves the generation of active metabolites, ie, EXP3179 or EXP3174. Based on recent observations which revealed various potentially vasoprotective effects of EXP3179, we hypothesized that EXP3179 might possess an individual pharmacological potency, which may not only contribute to the beneficial effects of LOS but also qualify EXP3179 as an independent lead for future treatment strategies to prevent acute atherothrombosis. Here, we demonstrate that EXP3179 selectively reduces collagen-dependent human platelet activation, adhesion, and aggregation independent of AT1-receptor antagonism. In this regard, Kalinowski et al already reported that application of LOS itself impairs collagen-induced platelet aggregation and adhesion, which was attributed to a release of NO. Indeed, Watanabe et al recently identified EXP3179 as a potent activator of the eNOS phosphorylation. In platelets, however, the role of eNOS remains controversially discussed. Furthermore, eNOS-associated signaling involves not only collagen-related but also alternative pathways, eg, thrombin or ADP, both of which were not seen to be influenced by EXP3179 in the present study. Instead, we show that EXP3179 inhibited GPVI receptor–mediated platelet aggregation and activation as determined by platelet PAC-1 expression. Furthermore, EXP3179—in contrast to the main LOS metabolite EXP3174—substantially reduced the adhesion of GPVI-receptor expressing CHO cells on collagen-I under shear conditions confirming the specific impact of EXP3179 on GPVI-receptor function. Although the relevance of GPVI-receptor activation in experimental murine arterial thrombosis has lately been critically discussed, recent observations by Penz et al again emphasized the importance of collagen-dependent platelet activation via the GPVI-receptor for acute thrombus formation at sites of human atherosclerotic lesions. Therefore, we investigated the direct impact of EXP3179 on platelet aggregation induced by human atheromatous plaque material. Here we show that EXP3179 inhibits thrombus formation after stimulation with human plaque material. To evaluate the effect of EXP3179 on platelet adhesion and aggregation in vivo we used an established mouse model of carotid injury. In this regard, we and others reported that murine thrombus formation in this model crucially depends

**Figure 4.** Impact of EXP3179 on atherosclerotic plaque material-induced platelet aggregation. Incubation of human platelets with EXP3179 led to a significant reduction of atherosclerotic plaque material-induced platelet aggregation in comparison to administration of solvent analyzed by optical aggregometry. Data are given as maximal aggregation in %. Data are mean±SD; *P<0.01 vs solvent; n=4.

**Figure 5.** Effects of EXP3179 on murine platelet aggregation in vitro and platelet adhesion in vivo. A, Incubation of murine PRP with solvent or EXP3179 (500 μmol/L) reduced collagen-I–induced platelet aggregation analyzed by optical aggregometry. PRP was pooled, using PRP of 8 animals for n=3 experiments. Data are mean±SD; *P<0.01 vs solvent; n=3. B, Intravital fluorescent microscopy after ligature of the common carotid artery showed a reduction of murine platelet adherence at the site of injury after preincubation of murine PRP with EXP3179 in comparison to solvent administration. Data are mean±SD; *P<0.01 vs solvent; n=6.
on GPVI-receptor activation. Here we show that EXP3179 substantially reduces platelet adhesion in response to vessel injury underlying the impact on GPVI receptor–mediated platelet activation by EXP3179 also in vivo.

Study Limitations and Clinical Perspectives

Before the onset of the study presented, the pharmacokinetics and dynamics of EXP3179 in vivo had only been investigated once and in context of a single orally-applied dosage of LOS. Thus, we are the first to consider and apply EXP3179 as an individual potential drug. Although we did not observe any signs of acute intoxication or unspecificity (compared with solvent) when using EXP3179 at a concentration of 500 μmol/L, further studies will be needed to establish a dose-dependent pharmacological profile of EXP3179. To summarize, this study describes EXP3179 as a potent inhibitor of collagen-dependent platelet activation, aggregation, and adhesion via GPVI in vitro and in vivo. These results indicate EXP3179 as an individual platelet-inhibitory and potentially vasoprotective agent in the experimental attempt to identify new therapeutic strategies to prevent fatal atherothrombotic cardiovascular events.

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References

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Figure I

**Figure I**

Comparison of PAC-1 expression (mean intensity of immunofluorescence) under different conditions:

- **Top Panel:**
  - Unstimulated PRP, Stimulation PRP, Solvent, EXP3179 (10 µM), EXP3179 (100 µM), EXP3179 (500 µM)
  - Collagen-I concentrations: 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL
  - Statistical significance indicated by symbols: +, n.s.

- **Middle Panel:**
  - Unstimulated PRP, Stimulation PRP, Solvent, LOS (10 µM), LOS (100 µM), LOS (500 µM)
  - Statistical significance indicated by symbols: +, n.s., #

- **Bottom Panel:**
  - Unstimulated PRP, Stimulation PRP, Solvent, EXP3174 (10 µM), EXP3174 (100 µM), EXP3174 (500 µM)
  - Statistical significance indicated by symbols: +, n.s., #
Figure II

0.1 µg/mL 4c9
0.2 µg/mL 4c9
0.5 µg/mL 4c9

PAC-1 expression (mean intensity of immunofluorescence)

unstim PRP  |  stim PRP  |  solvent  |  EXP3179 10 µM  |  EXP3179 100 µM  |  EXP3179 500 µM

**  |  **  |  n.s.  |  +  |  *

unstim PRP  |  stim PRP  |  solvent  |  LOS 10 µM  |  LOS 100 µM  |  LOS 500 µM

**  |  **  |  n.s.  |  +  |  *

unstim  |  PRP  |  solvent  |  EXP3174 10 µM  |  EXP3174 100 µM  |  EXP3174 500 µM

0  |  100  |  200  |  300  |  400  |  500  |  600

n.s.  |  +  |  *
Figure III
adherent cells on collagen-I/mm²

Figure IV
Figure V
Figure VI

The graph shows the number of adherent cells on collagen-I/mm² for different treatments.

- CHO-F
- CHO-F + solvent + EXP3179
- CHO-F + solvent + LOS
- CHO-F + LOS
- CHO-F + solvent + EXP3174
- CHO-F + EXP3174
**Figure I:** Dose-dependent Inhibition of collagen-I dependent human platelet activation by EXP3179, LOS and EXP3174. EXP3179, LOS and EXP3174 dose-dependently inhibited collagen-I dependent platelet activation measured by platelet PAC-1 expression using flow cytometry. Specific antibody binding was expressed as mean intensity of immunofluorescence. Data represent mean±SD, +p<0.05 vs unstimulated (unstim) PRP, #p<0.05 vs stimulated (stim) PRP or solvent, *p<0.01 vs stim PRP or solvent, n=4-7. No significant difference between solvent and stimulation of untreated PRP was observed.

**Figure II:** Dose-dependent Inhibition of GPVI-receptor dependent human platelet activation by EXP3179, LOS and EXP3174. EXP3179, LOS and EXP3174 dose-dependently inhibited GPVI-receptor dependent platelet activation measured by platelet PAC-1 expression after stimulation with the GPVI-receptor activating antibody 4c9 using flow cytometry. Specific antibody binding was expressed as mean intensity of immunofluorescence. Data represent mean±SD, +p<0.05 vs stimulated (stim) PRP or solvent, *p<0.01 vs stim PRP or solvent, **p<0.01 vs unstimulated (unstim) PRP, n=4-7. No significant difference between solvent and stimulation of untreated PRP was observed.
**Figure III:** Expression of the human GPVI-receptor on CHO-GPVI cells versus CHO-F cells. Successful transfection and expression of the human GPVI-receptor on CHO-GPVI cells in comparison to non-transfected CHO-F cells was measured by flow cytometry using 5C4, an anti-GPVI receptor antibody. Specific staining was controlled by using antibody-matched isotype.

**Figure IV:** Adhesion of CHO-GPVI cells on collagen-I under constant shear conditions after pre-incubation with the appropriate solvents (500 μM) of EXP3179, LOS or EXP3174. Data represent mean±SD.

**Figure V:** Adhesion of CHO-GPVI cells on collagen-I under constant shear conditions after pre-incubation with Irbesartan (IRB) or appropriate solvent (500 μM). Data represent mean±SD.

**Figure VI:** Adhesion of control CHO cells on collagen-I under constant shear conditions after pre-incubation with EXP3179, LOS, EXP3174 or appropriate solvent (500 μM). Data represent mean±SD.