

Specific inhibition of the transporter MRP4/ABCC4 affects multiple signaling pathways and thrombus formation in human platelets

Robert Wolf,^{1,2} Sophie Grammbauer,¹ Raghavendra Palankar,³ Céline Tolksdorf,^{1,4} Eileen Moritz,^{1,2} Andreas Böhm,¹ Mahmoud Hasan,¹ Annika Hafkemeyer,¹ Andreas Greinacher,³ Mladen V. Tzvetkov,¹ Bernhard H. Rauch^{1,2,4#} and Gabriele Jedlitschky^{1#}

¹Department of General Pharmacology, Center of Drug Absorption and Transport (C_DAT), University Medicine Greifswald, Greifswald; ²German Center for Cardiovascular Research (DZHK), Partner Site Greifswald; ³Department of Immunology and Transfusion Medicine, University Medicine Greifswald, Greifswald and ⁴Department of Human Medicine, Section of Pharmacology and Toxicology, Carl von Ossietzky University of Oldenburg, Oldenburg, Germany

[#]BHR and GJ contributed equally as co-senior authors.

Correspondence: G. Jedlitschky
gabriele.jedlitschky@med.uni-greifswald.de

Received: August 4, 2021.

Accepted: March 9, 2022.

Prepublished: March 17, 2022.

<https://doi.org/10.3324/haematol.2021.279761>

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Abstract

The multidrug resistance protein 4 (MRP4) is highly expressed in platelets and several lines of evidence point to an impact on platelet function. MRP4 represents a transporter for cyclic nucleotides as well as for certain lipid mediators. The aim of the present study was to comprehensively characterize the effect of a short-time specific pharmacological inhibition of MRP4 on signaling pathways in platelets. Transport assays in isolated membrane vesicles showed a concentration-dependent inhibition of MRP4-mediated transport of cyclic nucleotides, thromboxane (Tx)B2 and fluorescein (FITC)-labeled sphingosine-1-phosphate (S1P) by the selective MRP4 inhibitor Ceefourin-1. In *ex vivo* aggregometry studies in human platelets, Ceefourin-1 significantly inhibited platelet aggregation by about 30-50% when ADP or collagen was used as activating agents, respectively. Ceefourin-1 significantly lowered the ADP-induced activation of integrin α IIb β 3, indicated by binding of FITC-fibrinogen (about 50% reduction at 50 μ M Ceefourin-1), and reduced calcium influx. Furthermore, pre-incubation with Ceefourin-1 significantly increased PGE₁- and cinaciguat-induced vasodilator-stimulated phosphoprotein (VASP) phosphorylation, indicating increased cytosolic cAMP as well as cGMP concentrations, respectively. The release of TxB2 from activated human platelets was also attenuated. Finally, selective MRP4 inhibition significantly reduced both the total area covered by thrombi and the average thrombus size by about 40% in a flow chamber model. In conclusion, selective MRP4 inhibition causes reduced platelet adhesion and thrombus formation under flow conditions. This finding is mechanistically supported by inhibition of integrin α IIb β 3 activation, elevated VASP phosphorylation and reduced calcium influx, based on inhibited cyclic nucleotide and thromboxane transport as well as possible further mechanisms.

Introduction

The multidrug resistance protein 4 (MRP4) (ABCC4) is a member of the MRP/CFTR subfamily (C-branch) of the ATP-binding cassette (ABC) transporters, a family of proteins that mediate an ATP-driven transmembrane transport of compounds. It represents a very versatile transporter, which is expressed in several tissues with high amounts in platelets.¹⁻⁴ Its substrate spectrum covers several drugs, namely nucleoside-based antiviral and anti-cancer agents but interestingly also a number of endogenous signaling molecules. These include primarily the cyclic nucleotides cAMP and cGMP.^{5,6} MRP4 has been established as an inde-

pendent regulator of intracellular cAMP levels and of cell proliferation and differentiation in several cell types, including vascular smooth muscle cells as well as hematopoietic cells.^{7,8} Furthermore, cyclic nucleotides play a major role in platelet activation and regulation. In MRP4-deficient mice, dysregulation of platelet cAMP homeostasis was observed.⁹⁻¹¹ This dysregulation may be due to reduced cAMP efflux and/or intracellular sequestration since the exact localization of MRP4 in resting platelets has yet to be clarified. There is evidence for plasma membrane localization,¹⁰ but also for a partial intracellular localization of MRP4 in association with the dense granule markers.^{1-3,11} In addition, Cheepala *et al.* reported a reduced plasma membrane lo-

calization of the major collagen receptor GPVI and inhibition of collagen-induced platelet aggregation in their knockout mouse model.¹⁰ While the role of MRP4 in cAMP signaling is well established, its role in platelet cGMP homeostasis and other cAMP-independent pathways is less clear. MRP4 also transports lipid mediators such as eicosanoids and may directly mediate the export of thromboxane from platelets.^{12,13} In addition, we showed that MRP4 is also involved in the release of sphingosine-1-phosphate (S1P), a potent pro-inflammatory mediator, from platelets.¹⁴ Thus, MRP4 appears to be an essential factor in the paracrine function of platelets. Based on these findings, the transporter has emerged as a potential target to interfere with platelet function.^{1,3,6,9-11,14} MRP4 inhibitors may complement the currently used aggregation inhibitors, whereby especially platelet hyperreactivity as well as platelet-induced inflammatory processes may be reduced. Enhanced platelet reactivity has been linked to MRP4 overexpression in cases of aspirin resistance^{3,15,16} as well as in patients infected with the human immunodeficiency virus (HIV).¹⁷ The present study aimed to comprehensively characterize the effect of a selective MRP4 inhibitor on different mediators and signaling pathways in platelets, thereby evaluating the impact of a short-term pharmacological MRP4 inhibition on the function of human platelets. In previous studies, often rather unspecific inhibitors such as the leukotriene receptor antagonist MK571¹⁸ were used to inhibit MRP4.^{19,20} Meanwhile, more selective inhibitors of MRP4, namely Ceefourin-1,²¹ have become available. Effects on thrombus formation were also studied in a microfluidic flow chamber model to mimic physiological shear stress in both whole human blood and in samples from MRP4-deficient compared to control mice.

Methods

More details are provided in the *Online Supplementary Appendix*.

Human blood samples and animals

Human venous blood was taken from healthy volunteers after written informed consent according to the Declaration of Helsinki and approval from the Institutional Ethics Committee. Mrp4-deficient (Mrp4 (-/-)) mice were kindly provided by the late Dr. Gary D. Kruh (Cancer Center, University of Illinois, Chicago, IL, USA) and were maintained and backcrossed to C57BL/6 wild-type (WT) animals at the animal facility of the University Medicine Greifswald. Murine blood was obtained by right ventricular heart puncture.

Light transmission aggregometry and platelet thromboxane release

For aggregometry, platelet-rich plasma (PRP) was pre-

pared from human citrate blood as described¹⁴ and pre-incubated with Ceefourin-1, aspirin or cinaciguat as stated in the figure legends. For measurement of thromboxane release, washed platelets were pre-incubated with Ceefourin-1 and activated with collagen-related peptide CRP-XL and thrombin receptor-activating peptide PAR1-AP (15 minutes [min]). Concentrations of TxB2 in separated platelets and supernatants were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Flow cytometric analyses

Fibrinogen binding to integrin $\alpha IIb\beta 3$ - PRP was diluted in HEPES-buffered saline (HBS), pre-incubated with Ceefourin-1 (10-50 μ M) (15 min) and stimulated with either CRP-XL or ADP (10 min), before FITC-labeled human fibrinogen and R-phycoerythrin anti-human-CD62P were added (for supplier see the *Online Supplementary Appendix*). After 20 min, samples were fixed in 0.2% formaldehyde and subjected to flow cytometric acquisition.

VASP phosphorylation - Washed platelets were resuspended in HBS and pre-incubated with either Ceefourin-1 (50 μ M) alone or with Ceefourin-1 added to PGE₁ or cinaciguat (0.5-1 μ M). Platelets were fixed with formaldehyde and permeabilized with Triton-X100. Phospho-specific antibodies either against serine residue 157 or serine residue 239 of vasodilator-stimulated phosphoprotein (VASP) and the respective Alexa Fluor 488-conjugated secondary antibodies were added for flow cytometric analysis.

Calcium measurements

Washed platelets were incubated with Fura-2 AM and subjected to ratiometric calcium analysis using a fluorescence spectrophotometer (excitation 340/380 nm, emission 510 nm).

Flow chamber experiments

Parallel channel flow chambers (ibidi μ -slide VI 0.1, Gräfelting, Germany) were coated with Horm collagen (Takeda Pharmaceutical, Berlin, Germany). Human or murine blood was anticoagulated with hirudin (525 ATU/mL) and heparin (5 IU/mL) or with PPACK (Cayman Chemical, Ann Arbor, MI, USA) (400 μ M) and hirudin, respectively. Platelet specific antibodies (FITC-labeled anti-human CD42a or DyLight 488-conjugated anti-mouse GPIIb β) (for supplier see the *Online Supplementary Appendix*) were added and blood was incubated with Ceefourin-1 or the respective solvent at 37°C. Blood was perfused through the microchannels under high arterial shear conditions (1,800⁻¹) for 5 min. After completion of the experiment, two images at the beginning, two at the end and one in the center of the channel were obtained, using a confocal laser scanning microscope (Carl Zeiss LSM 780, Oberkochen, Germany) (40x objective). Size of thrombi and surface area coverage

were analyzed with ImageJ²² software. Image segmentation was performed in Bitplane Imaris version 7.65. (Oxford Instruments, Abingdon, UK) using the surfaces creation wizard algorithm.²³ All flow experiments were performed according to International Society on Thrombosis and Hemostasis Scientific and Standardization Committee (ISTH SSC) recommendations.²⁴

Results

Ceefourin-1 effectively inhibits MRP4-mediated transport of several signaling compounds *in vitro*

In order to evaluate how effectively Ceefourin-1 interferes with the direct ATP-dependent transport of several signaling compounds, transport assays using inside-out membrane vesicles containing recombinant human MRP4 were performed. ATP-dependent transport of ³H-labeled cGMP was inhibited with a half maximal inhibitory concentration (IC₅₀) value of 5.7 μM, indicating that the transport of cyclic nucleotides is affected by Ceefourin-1 with high affinity (*Online Supplementary Figure S1A*). Also the more lipophilic substrates TxB2 and S1P were actively transported by MRP4, confirming previous studies.^{13,14} TxB2 transport could be blocked by Ceefourin-1 with nearly the same *in vitro* potency (IC₅₀: 3.6 μM) as cGMP transport (*Online Supplementary Figure S1B*). However, higher concentrations of Ceefourin-1 were required to interfere with S1P transport (IC₅₀ of about 50 μM) (*Online Supplementary Figure S1C*).

MRP4 inhibition reduces the release of thromboxane from human platelets

Since the ATP-dependent TxB2 transport was potently inhibited by Ceefourin-1 in the membrane vesicle assay, the impact of MRP4 inhibition on thromboxane release by stimulated human platelets *ex vivo* was further investigated. For this, incubation of the platelets with Ceefourin-1 was performed prior to the addition of CRP-XL or PAR1-AP, which activate collagen or thrombin receptors, respectively. Subsequently, concentrations of TxB2 (the stable metabolite of TxA2) were measured in platelet supernatants and the respective pellets by LC-MS/MS. In order to validate the results, we compared this method with an established enzyme-linked immunosorbent assay (ELISA) and found only a negligible inter-method variability (*Online Supplementary Figure S2*). Basal TxB2 release in control platelets was 2.27±0.39 pg/10⁶ platelets and increased strongly upon stimulation with CRP-XL (1 μg/mL) and PAR1-AP (50 μM) to 5.96±1.56 pg/10⁶ and 5.91±1.64 pg/10⁶ platelets, respectively. In comparison, pre-treatment with Ceefourin-1 led to a significant decrease in basal as well as CRP-XL- and PAR1-AP-induced TxB2 release (Figure 1A). In addition, also the total amount of TxB2 (supernatant and pellet combined) was significantly re-

duced by Ceefourin-1 in CRP-XL- and PAR1-AP-treated samples (Figure 1B). This indicates that TxB2 formation is also affected by MRP4 inhibition. However, further analyses of our data revealed that the relative TxB2 release, which was calculated from the fraction released divided by the total amount, was still significantly diminished. In platelets stimulated with CRP-XL (1 μg/mL) 35.8±3.3% were released in the presence of Ceefourin-1 (vs. control: 45.0±1.5%) and with PAR1-AP (50 μM) 36.4±4.7% (vs. control: 48.0±7.8%) (Figure 1C). This suggests that Ceefourin-1 attenuates platelet TxB2 release via a diminished TxB2 synthesis during activation combined with a direct effect on the TxB2 transport across the plasma membrane.

Ceefourin-1 treatment impairs platelet aggregation in human and murine PRP

Light transmission aggregometry with different stimuli was performed to investigate, whether short-time exposure of PRP to Ceefourin-1 leads to impaired platelet aggregation. MRP4 inhibition resulted in a reduction of maximum platelet aggregation, with the most prominent effect (about 50% inhibition at 10 μM Ceefourin-1) being observed with the strong agonist collagen (5 μg/mL) (38.3±10.3% aggregation vs. 77.3±4.0% for the solvent control) (Figure 2). Although less pronounced, a significant effect on aggregation was also observed in ADP- and PAR1-AP-stimulated platelets (27% and 13% reduction at 10 μM Ceefourin-1, respectively), while the synthetic thromboxane analog U46619 had no significant effect. In comparison, aspirin was used to block thromboxane synthesis, leading, as expected, to a reduced aggregation with the most pronounced effect also with collagen-induced activation (63% at 30 μM aspirin). When both compounds were combined, only a tendency towards an additive effect was observed, which, however, was not statistically significant. These results further substantiate the finding of MRP4 being involved in the release of thromboxane, not excluding its potential role in intrinsically controlling platelet activation and, therefore, thromboxane production.

Since several MRP4-inhibiting compounds show significant off-target effects, we performed platelet aggregation experiments with PRP from WT and *Mrp4*-deficient mice to verify the selectivity of Ceefourin-1. In line with previous studies,^{9,10} the *Mrp4* knockout led to an impaired aggregation response to collagen stimulation (30.7±5.7% vs. 46.7±3.8% aggregation with 10 μg/mL collagen) (Figure 2C). A similar reduction was achieved by the treatment with Ceefourin-1 in WT platelets (31.3±3.9% vs. 46.7±3.8%). However, Ceefourin-1 resulted in no further attenuation of aggregation in the *Mrp4*-deficient platelets, indicating that the effect of Ceefourin-1 on platelet function is only due to MRP4 inhibition. Ceefourin-1 also reduced platelet aggregation after stimulation with ADP only in the WT platelets (*Online Supplementary Figure S3*).

In addition, we evaluated the effect of Ceefourin-1 on platelet viability using an assay based on the intracellular calcein accumulation as described in the *Online Supplementary Appendix*. Ceefourin-1 in concentrations of up to 50 μM had no significant effect on platelet viability compared to the solvent control (*Online Supplementary Figure S4*).

Platelet fibrinogen binding and calcium influx is inhibited by Ceefourin-1

The impact of Ceefourin-1 on different sub-aspects of platelet activation, like integrin $\alpha\text{IIb}\beta\text{3}$ activation and α -granule release were assessed by flow cytometric analyses of fluorescence-labeled fibrinogen and anti-CD62P antibody binding. Fibrinogen binding was significantly reduced by Ceefourin-1 ($80.9\pm 4.9\%$ and $75.3\pm 4.0\%$ of control with 30 μM and 50 μM Ceefourin-1, respectively) at a sub-maximal concentration of CRP-XL (Figure 3A). ADP-induced fibrinogen binding was also markedly abrogated to $47.1\pm 7.3\%$ of control with 50 μM Ceefourin-1 (Figure 3B). Higher concentrations of the agonists led to a less pronounced effect of MRP4 inhibition, which disappeared completely at the highest concentrations used. Additionally, we found CD62P surface exposure to be significantly reduced upon stimulation with ADP but not with CRP-XL (Figure 3C and D).

Since calcium is essential for the change in conformation of integrin $\alpha\text{IIb}\beta\text{3}$, allowing it to bind fibrinogen, we measured the increase of free cytosolic calcium in Fura-2-loaded human platelets (Figure 4A to C). In order to discriminate between calcium influx and mobilization from intracellular stores, platelets were activated in the presence (left panels) or absence (right panels) of external calcium. Blocking MRP4 entails a reduction in the area under the fluorescence curve (area under the curve [AUC]) upon stimulation with ADP, resulting predominantly from an impaired calcium influx (8.0 ± 0.5 vs. 11.4 ± 0.8 R(340/380)*s in calcium-containing medium). The signal in calcium-free medium, reflecting mobilization from intracellular stores, was markedly lower. However, there was also a trend towards reduced AUC values after incubation with Ceefourin-1 for the calcium mobilization, which became statistically significant when the decrease was calculated relative to the respective solvent control of each blood sample donor (separate experiment) (Figure 4C).

Ceefourin-1 enhances VASP phosphorylation and cyclic nucleotide-dependent platelet inhibition

The cyclic nucleotides cGMP and cAMP are important second messengers involved in platelet inhibition, especially by endothelium-derived factors, such as nitric oxide (NO) or prostacyclin (PGI₂). Both cGMP and cAMP are able to activate protein kinases (PK), leading to the phosphorylation and inhibition of proteins, which are involved in the signaling for platelet activation. In order to

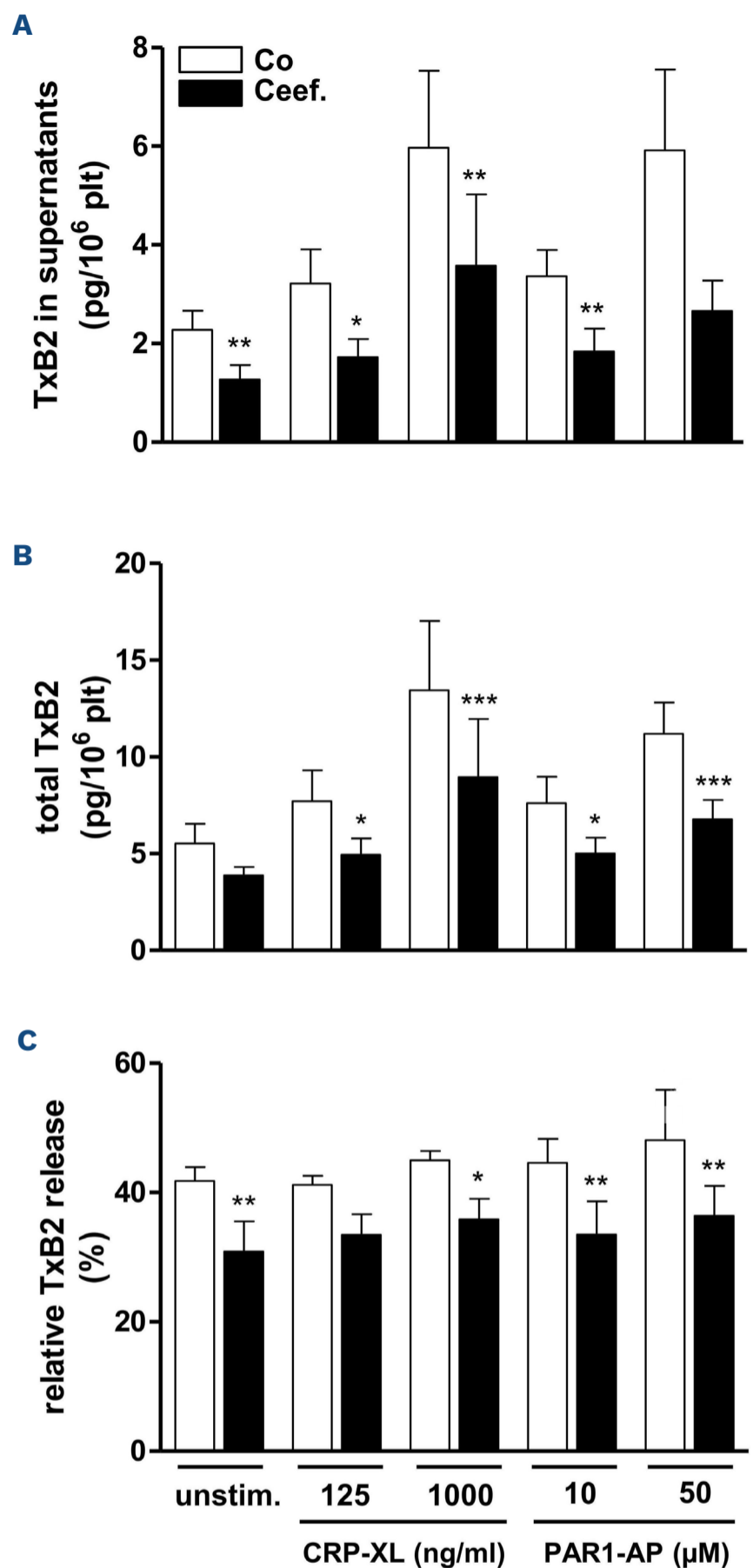


Figure 1. Inhibition of TxB2 release from human platelets by Ceefourin-1. Washed platelets were incubated for 15 minutes at 37°C with Ceefourin-1 (Ceef., 50 μM , black bars) or the respective solvent control (Co, 0.5% dimethyl sulfoxide [DMSO], white bars) and then either left unstimulated (unstim.) or activated with collagen-related peptide CRP-XL (125 ng/mL and 1,000 ng/mL) and the thrombin receptor-activating peptide PAR1-AP (10 μM and 50 μM). After 15 min, the platelets were pelleted and TxB2 was analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the supernatants and pellets. (A and B) Absolute TxB2 concentrations in platelet supernatants (A) and in platelet pellets (B) in pg/1x10⁶ platelets (plt). (C) Platelet TxB2 release in % of the total amount formed. Values represent means + standard error of the mean from n=6 different donors. * $P < 0.05$; ** $P < 0.01$ vs. the respective solvent control.

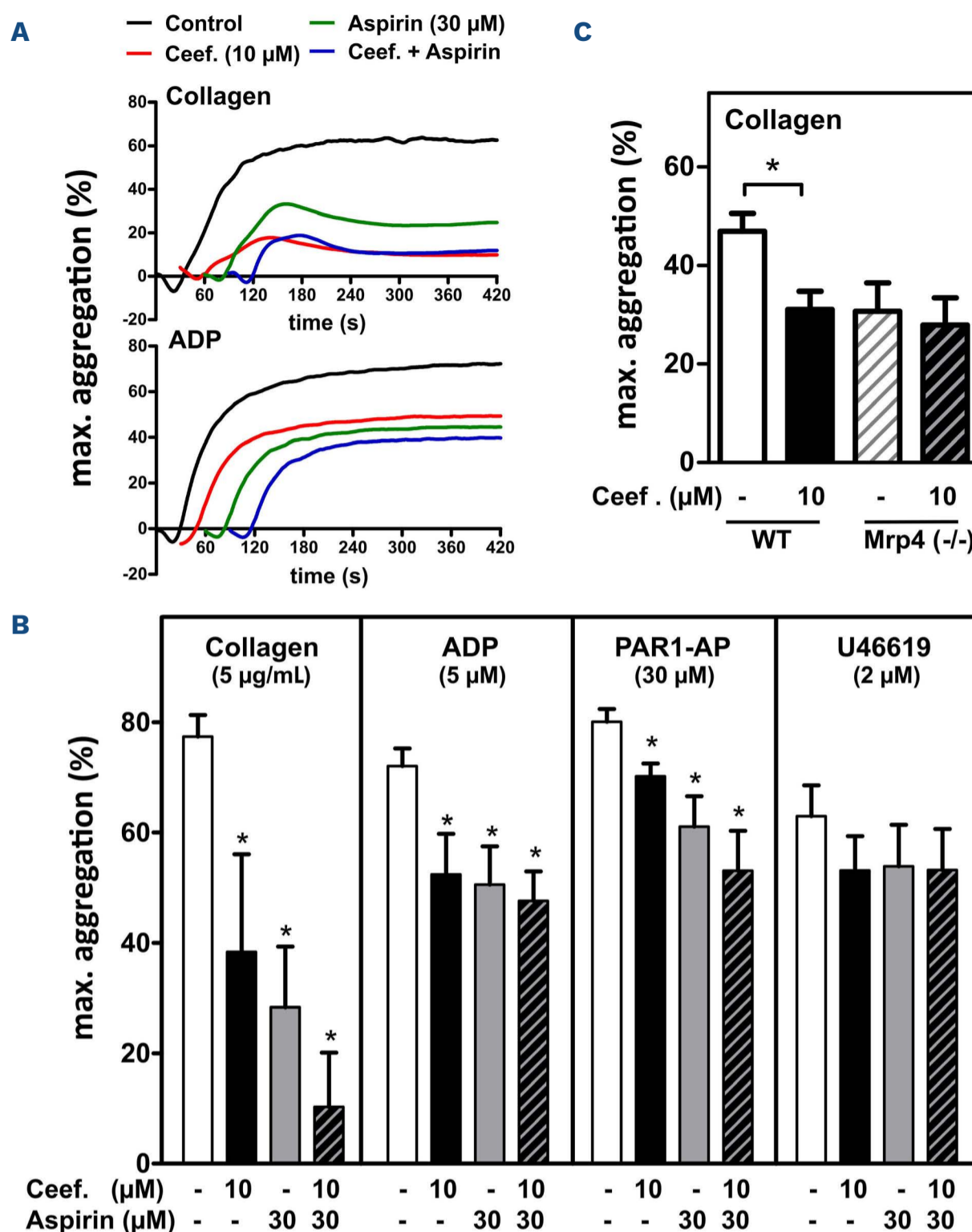


Figure 2. Effect of Ceefourin-1 on platelet aggregation ex vivo. (A and B) Human platelet-rich plasma (PRP) was pretreated with either only the solvent (control, (-)), or Ceefourin-1 (Ceef.; 10 μ M for 20 minutes), or aspirin (30 μ M; for 5 minutes), or the combination of both, and then stimulated with the following agonists: collagen (5 μ g/mL), ADP (5 μ M), PAR1-AP (30 μ M) or U46619 (2 μ M). Platelet aggregation was determined by light transmission aggregometry. Representative curves of platelet aggregation stimulated by collagen and ADP are given in (A). The inhibition of the maximal aggregation is summarized in (B) (means + standard error of the mean [SEM]; n=6-11; * P <0.05 vs. control). (C) PRP (diluted 1:2 with Tyrode's buffer) from age- and sex-matched wild-type (WT) or Mrp4-deficient (Mrp4(-/-)) mice was pretreated with either only the solvent (control, (-)) or Ceefourin-1 (Ceef.; 10 μ M) and then stimulated with collagen (10 μ g/mL). Aggregation curves were monitored and the maximal extent of aggregation (%) was calculated (mean + SEM; n=5-7). Ceefourin-1 led to a significant reduction of the aggregation only in WT platelets (* P <0.05).

determine cytosolic cyclic nucleotide levels in human platelets, we measured the phosphorylation of VASP at two different serine residues (ser-157 and ser-239) by flow cytometry. The cAMP-elevating agent PGE₁ profoundly increased VASP phosphorylation at ser-157, the preferred phosphorylation site of PKA. Pre-incubation with Ceefourin-1 (50 μ M) significantly increased this effect (310.4±25.3% vs. 211.3±14.7% with 1 μ M PGE₁) (Figure 5A, left panel). Note that Ceefourin-1 alone only tends to

elevate background VASP phosphorylation. The phosphorylation of ser-239, the preferred substrate of PKG, is similarly elevated in the presence of cinaciguat, an activator of soluble guanylate cyclase. As shown in Figure 5A, right panel, the specific inhibition of MRP4 resulted in a 1.8-fold increase in the cinaciguat-stimulated VASP phosphorylation.

In order to investigate whether MRP4 inhibition can enhance cGMP-mediated effects in platelets, we measured

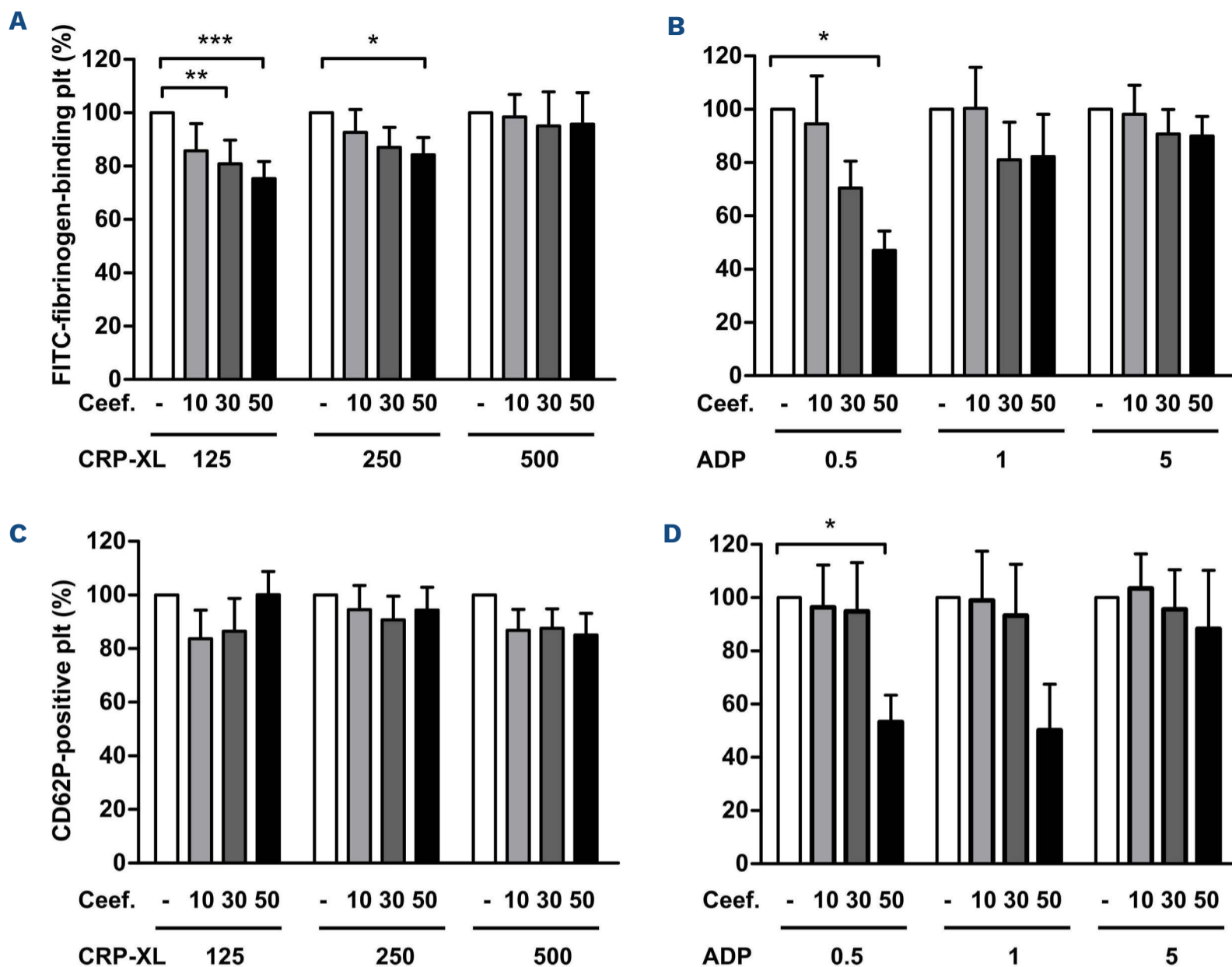


Figure 3. Effect of MRP4 inhibition on platelet fibrinogen binding and CD62P surface exposure. Binding of FITC-labeled fibrinogen (A and B) and R-phycoerythrin-labeled anti-CD62P antibody (C and D) to platelets (plt) was assessed by flow cytometry as a measure for integrin $\alpha\text{IIb}\beta\text{3}$ activation and α -granule release, respectively. Platelet-rich plasma was preincubated with Ceefourin-1 (Ceef.) (10–50 μM) or the solvent control (0.5% dimethyl sulfoxide [DMSO]) for 15 minutes (min) and then stimulated with the collagen-related peptide CRP-XL (125–500 ng/mL) (A and C) or ADP (0.5–5 μM) (B and D) for 10 min before FITC-fibrinogen or anti-CD62P antibody were added. After fixation in formaldehyde the samples were subjected to flow cytometric analysis. Data are given in % of the respective solvent control (means + standard error of the mean, $n=4-6$ platelet-rich plasma samples of different donors, performed in duplicates). Significant differences with $*P<0.05$, $**P<0.01$, and $***P<0.005$ vs. the respective solvent control.

platelet aggregation in the presence of cinaciguat (0.1 μM) and Ceefourin-1 (50 μM). A 3-minute pre-incubation with either substance alone led to only a modest reduction of maximum platelet aggregation as well as aggregation slope (Figure 5B). However, both substances combined significantly decreased the magnitude and slope of platelet aggregation to 37% and 46% of control, respectively.

Specific inhibition of MRP4 reduces platelet adhesion and thrombus formation under flow

In order to investigate whether the effect of Ceefourin-1 on platelet aggregation is also relevant under shear conditions, whole blood with FITC-anti-CD42a-labeled platelets was perfused through collagen-coated microchannels under high arterial shear conditions. Platelet

adhesion and thrombus formation were analyzed. Spiking whole blood with Ceefourin-1 (50 μM) resulted in a significant reduction in the area of platelet thrombi ($29.9\pm 1.4 \mu\text{m}^2$ vs. $18.4\pm 0.8 \mu\text{m}^2$) as well as in the total area of the channel, which was covered by thrombi (Figure 6A and B). We verified the selectivity of Ceefourin-1 again in this assay by conducting perfusion experiments with freshly taken samples of whole blood from MRP4-deficient and WT control mice. As shown in Figure 6C and D, the average area of thrombi was reduced in blood from MRP4 (-/-) mice by about 45% as well as after incubation of blood from WT animals with Ceefourin-1 *ex vivo* as compared to control (WT without Ceefourin-1). Strikingly, no inhibitory effect on thrombus formation was seen with Ceefourin-1 in blood from MRP4-deficient mice.

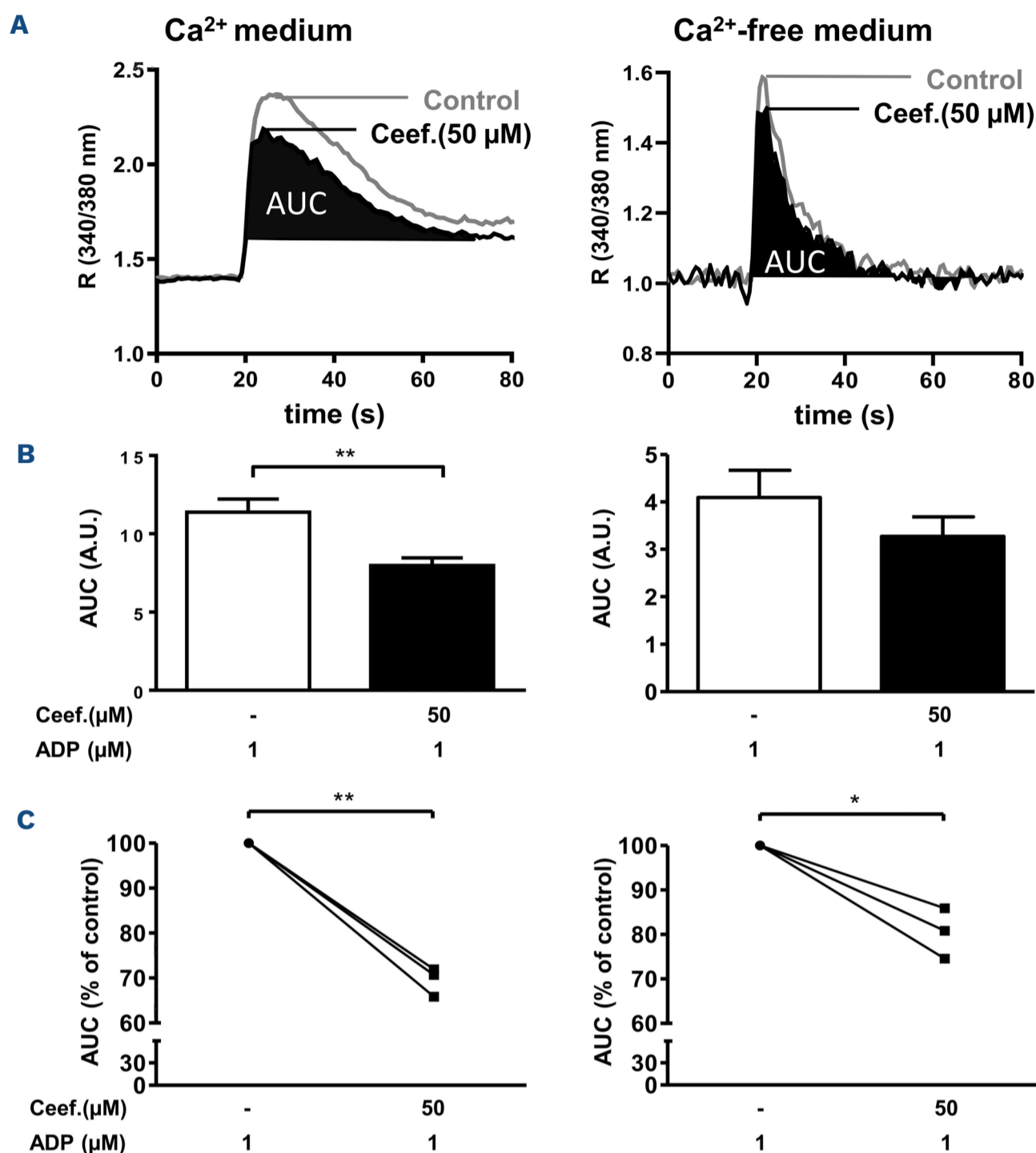


Figure 4. Effect of MRP4 inhibition on intracellular calcium levels in platelets. Cytosolic free calcium was measured spectrophotofluorometrically in Fura-2-loaded washed platelets. Measurements were performed either in Ca²⁺ medium (Tyrode's buffer with 2 mM CaCl₂) (left panels) or Ca²⁺-free medium (Tyrode's buffer with 0.2 mM EGTA) (right panels). In (A) the time-dependent increase in cytosolic calcium (indicated by R (340/380 nm)) after stimulation with ADP (1 μM) and the effect of Ceefourin-1 (50 μM) in representative experiments are depicted. In (B) the area under the fluorescence curve (area under the curve [AUC]) as a measure for the total change in calcium concentration was calculated (means + standard error of the mean from n=3 different donors, each measured in duplicates or quadruplicates). The reduction after Ceefourin-1 treatment is also given in % of the respective solvent control for each donor (each separate experiment) in (C) (significant differences with **P*<0.05, and ***P*<0.01).

Discussion

An impact of MRP4 on platelet function is indicated by several lines of evidence, including studies in *Mrp4* knockout mice^{9,10} and recent data from human individuals with a defect in the *ABCC4/MRP4* gene.⁴ MRP4 may affect different signaling pathways in human platelets through the transport of several compounds. In the present study, we used Ceefourin-1, which has been described as a selective MRP4 inhibitor²¹ to comprehensively characterize the effects on the function of human platelets that could be anticipated when applying a short-time pharmacological

inhibition of MRP4. It has been shown that Ceefourin-1 is highly selective for MRP4 over several ABC transporters, including other members of the MRP family such as MRP1 and MRP3 (ABCC1 and ABCC3), which are also expressed in platelets.² However, this does not exclude off-target effects of Ceefourin-1 on other structures in platelets. Therefore, we compared the effect of the compound in WT and *Mrp4*-deficient mice in the classic aggregometry studies and in the flow chamber experiments and observed significant effects on platelet function only when MRP4 is expressed. Possible unspecific effects of Ceefourin-1 on platelet viability were also tested and ruled

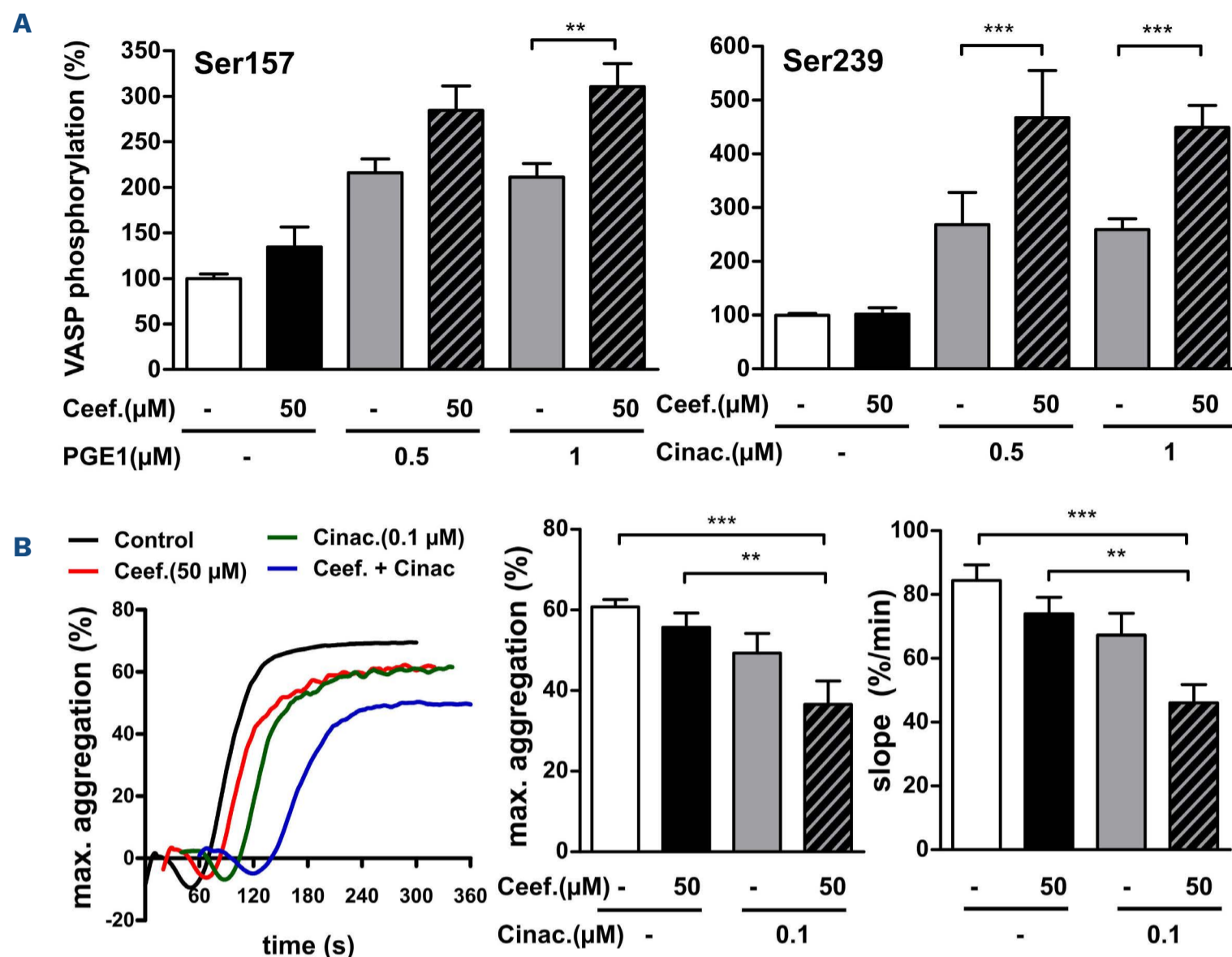


Figure 5. Effect of Ceefourin-1 on VASP phosphorylation (A) and cyclic nucleotide-dependent platelet inhibition (B). The phosphorylation of VASP at two different serine residues, serine-157 (the preferred substrate of PKA) (A, left panel) and serine-239 (the preferred substrate of PKG) (A, right panel) was determined by flow cytometry in human platelets as a measure for the cytosolic levels of cAMP and cGMP, respectively. Ceefourin-1 (Ceef., 50 μM) significantly amplified cAMP- and cGMP-dependent VASP phosphorylation, induced by PGE1 (1 μM) and cinaciguat (Cinac., 0.5 and 1 μM), respectively (means + standard error of the mean, n=6). (B) Platelet aggregation in the presence of cinaciguat (Cinac., 0.1 μM) and Ceefourin-1 (Ceef., 50 μM) was measured by light transmission aggregometry, to confirm the enhancement of cGMP-mediated effects by MRP4 inhibition. The aggregation was here stimulated by 1.25 μg/mL collagen. The left panel shows the aggregation curves of a representative experiment. Maximum platelet aggregation (middle panel) and the slope of platelet aggregation (right panel) were markedly decreased when both compounds were combined (means + standard error of the mean; n=20 measurements with platelet-rich plasma from 4 different donors).

out for the used concentrations. We further examined if there are substrate-dependent differences in the potency of Ceefourin-1 to inhibit the direct MRP4-mediated transport measured in inside-out membrane vesicles. Here, we observed that the MRP4-mediated transport of TxB2 is inhibited by Ceefourin-1 with a similar IC_{50} value as the transport of the cyclic nucleotide cGMP, although the structure of Ceefourin-1²¹ is more analogous to cyclic nucleotides than to eicosanoids. However, for inhibition of S1P transport, higher concentrations were required. This may indicate that the binding pocket for this substrate may be slightly different and Ceefourin-1 may not be the best compound to specifically interfere with the export or sequestration of this mediator. It should also be noted that the IC_{50} values determined in the inside-out membrane vesicles cannot directly be compared with the concentrations that are necessary to detect effects in intact

platelets. This is because only the proportion of the externally added Ceefourin-1 that has been taken up into the cells can inhibit the transporter at the cytosolic substrate binding site.

Thromboxane A2 (TxA2) is produced *de novo* upon activation of the platelets and amplifies the platelet response to a variety of stimulating agents. After its release, it is rapidly degraded to TxB2. Thromboxane has been supposed to diffuse through the platelet plasma membrane. However, *in vitro* assays in isolated membrane vesicles indicate that the presence of an ATP-dependent export pump is required for an effective export.¹³ Therefore, we investigated the impact of MRP4 inhibition on thromboxane release by stimulated human platelets in more detail. Ceefourin-1 pretreatment significantly reduced the thromboxane release from platelets after stimulation of the collagen or thrombin receptor. Thereby, the reduction in

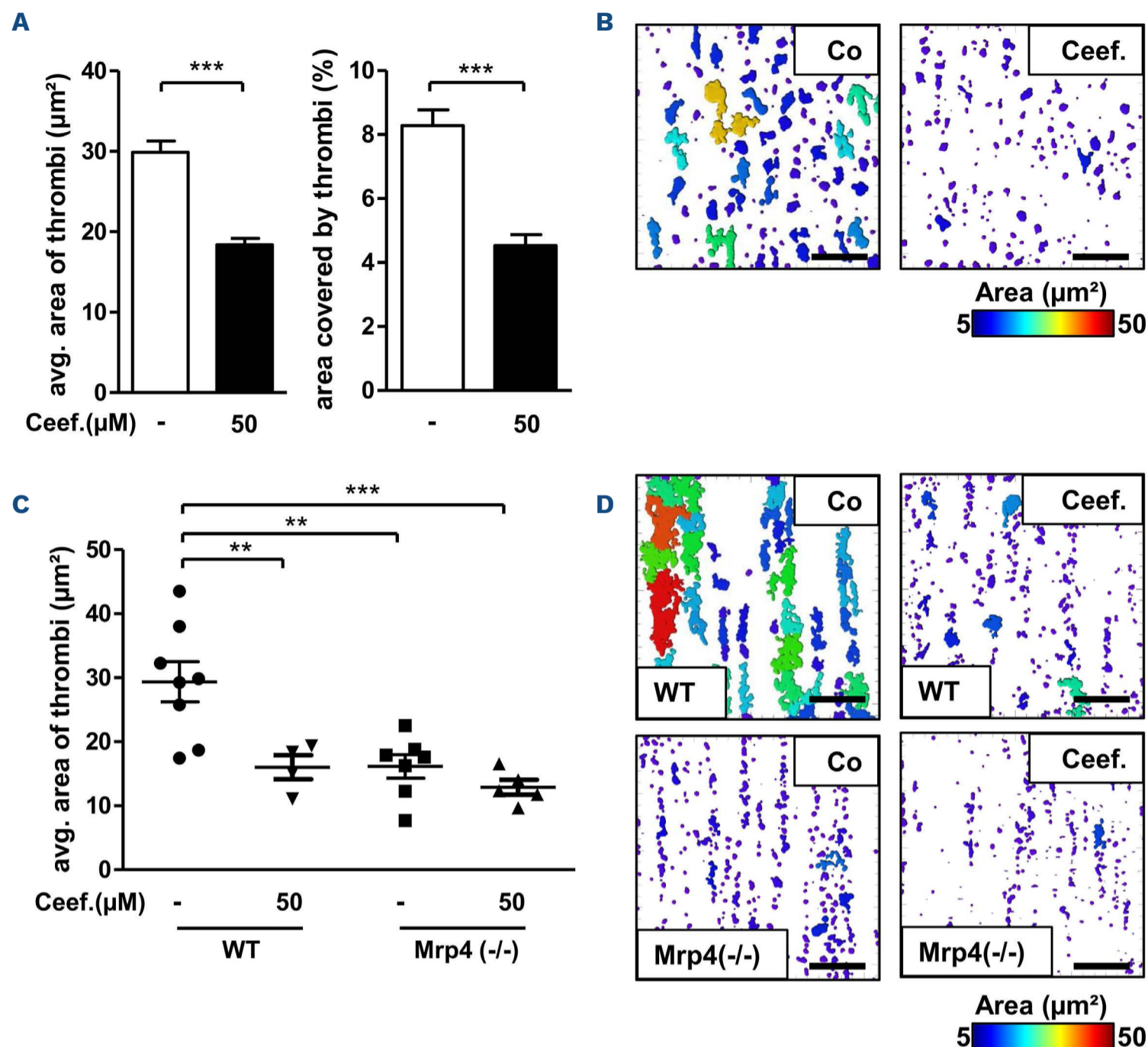


Figure 6. Role of MRP4 in platelet adhesion and thrombus formation under flow. (A and B) Whole human blood with FITC-anti-CD42a-labeled platelets (from $n=3$ different donors) was pre-incubated with Ceefourin-1 (Ceef., $50 \mu\text{M}$) or the respective solvent (Co, 0.5% dimethyl sulfoxide [DMSO]) and perfused through collagen-coated microchannels under high arterial shear conditions ($1,800\text{-}^{-1}$) for 5 minutes. Micrographs were taken at the beginning, the end, and in the center of the channel using a confocal laser scanning microscope (Zeiss LSM 780) with a $40\times$ objective. The average thrombus area (A, left panel) and surface area coverage by thrombi (A, right panel; means + standard error of the mean [SEM]) were analyzed with the ImageJ software. In (B) representative images are shown. (C and D) The effect of Ceefourin-1 was also evaluated in perfusions performed with whole blood from age- and sex-matched wild-type (WT) or $\text{Mrp4}(-/-)$ mice (with X488-labeled platelets). The average thrombus area (C, means \pm SEM and values of individual animals; $n=4-8$) was markedly reduced in $\text{Mrp4}(-/-)$ and Ceefourin-1-treated WT mice with no significant additional Ceefourin-1 effect in $\text{Mrp4}(-/-)$ animals. (D) Representative micrographs. Scale bars correspond to $50 \mu\text{m}$.

thromboxane release seems to be a combined effect of a diminished thromboxane synthesis during activation and a direct impact on the transport across the plasma membrane, since not only the total amount of this mediator but also the relative percentage that was released, was reduced. The fact that we observed no significant effect of Ceefourin-1 on the aggregation induced by the synthetic thromboxane analog U46619 and only a tendency towards an additive effect with aspirin is in agreement with this assumption. However, the effect of Ceefourin-1 to reduce thromboxane release was rather small compared with the potent ability of aspirin to prevent thromboxane forma-

tion, which is required to effectively inhibit thromboxane-dependent platelet activation.²⁵ Therefore, this effect of Ceefourin-1 can be an additional factor but is unlikely to fully account for the observed inhibition of platelet activation in response to agonists such as ADP and collagen. We studied platelet aggregation after short-time exposure to Ceefourin-1 with different stimuli because the published aggregometry data are also inconsistent. While Cheepala *et al.*¹⁰ reported that diminished aggregation of platelets from Mrp4 knockout mice is specific for collagen and $\text{Mrp4}(-/-)$ platelets did not have any defect in aggregation with either ADP or thrombin, Decouture *et al.*⁹ ob-

served a significant decrease in ADP- and PAR-4-activating peptide-induced aggregation in their knockout model. In human platelets, an impact of MRP4 inhibition was reported mainly on collagen-induced platelet aggregation.^{10,15,16} However, in ABCC4/MRP4-negative individuals a significant decrease in platelet aggregation was not observed with collagen and ADP at high concentration (10 μ M), but at lower ADP concentrations of 2.5 μ M and 5 μ M.⁴ With Ceefourin-1, we observed a significant effect on aggregation when collagen (5 μ g/mL) was used, but also with ADP (5 μ M) or PAR1-AP (30 μ M). Detection of fibrinogen binding to the platelets as a measure for integrin α IIb β 3 activation affirmed the observation that MRP4 inhibition affects platelet reactivity most effectively when the activating stimuli were used at a low submaximal dose, while it does not interfere at maximal activating conditions. At low ADP concentrations, an effect on degranulation was also observed indicated by the reduced surface exposure of P-selectin (CD62P).

Inconsistent observations regarding the role of cGMP in the context of MRP4-mediated effects on platelet function have been reported. ATP-dependent transmembrane transport and export of cGMP in platelets were shown to be affected by MRP4 inhibitors.^{1,19} However, Decouture et al.⁹ reported that MRP4 appears not to interfere with platelet cGMP homeostasis in their murine model since they observed no difference in total and secreted cGMP in WT or Mrp4-deficient platelets pre-incubated with sodium nitroprusside and stimulated by a PAR4-activating peptide. A rise in platelet cGMP levels, e.g., induced by NO-mediated activation of the soluble guanylate cyclase, results in a downregulation of platelet-activating signaling pathways. In this study, we used the phosphorylation of VASP at ser-239 as an indicator of platelet cytosolic cGMP levels and cinaciguat²⁶ as an activator of the soluble guanylate cyclase. Here, Ceefourin-1 was able to significantly increase the cinaciguat-stimulated VASP phosphorylation as well as to enhance markedly the cinaciguat-induced inhibitory effects on platelet aggregation. Ceefourin-1 analogously increased the phosphorylation at ser-157 induced by the cAMP-elevating agent PGE₁. These results indicate that MRP4 inhibition can intensify both cAMP- and cGMP-mediated effects in platelets and thus the response to several endothelium-derived vasodilators such as cAMP-elevating prosta-glandins as well as the cGMP-elevating nitric oxide, even though Ceefourin-1 alone only slightly elevated the background levels of these mediators. VASP phosphorylation is one key factor in the inhibition of platelet aggregation, while G α i signaling leads to activation. Ca²⁺-dependent signaling pathways synergize with the G α i signaling in the activation of integrin α IIb β 3 and also play a key role in granule secretion from activated platelets. Therefore, we examined the effect of Ceefourin-1 on the free calcium

concentrations in platelets, both in the presence and the absence of extracellular calcium, to discriminate between calcium entry across the plasma membrane and release from intracellular stores in the dense tubular system. The results indicate that blocking MRP4 mainly affects the agonist-induced calcium influx but also to some extent the intracellular calcium mobilization through direct or indirect mechanisms.

It was also the question if these relatively moderate effects of a short-time pharmacological MRP4 inhibition on platelet activation are sufficient to affect platelet adhesion and thrombus formation under blood flow. Therefore, we also tested the impact of Ceefourin-1 in a microfluidic flow chamber model and perfused whole blood through collagen-coated microchannels under high arterial shear conditions. Such devices have been recognized as a valuable tool to mimic the anatomy of healthy and stenotic blood vessels.^{27,28} Here, we could also demonstrate that spiking human or WT murine blood with the MRP4 inhibitor significantly reduced the average thrombus size and the surface area covered by thrombi.

In conclusion, pharmacological inhibition of MRP4 affects several signaling pathways in platelets mechanistically based on the transport inhibition not only of cAMP but also cGMP as well as of the lipid mediators thromboxane and S1P. However, additional direct effects on alternative biochemical pathways cannot be excluded. MRP4-selective platelet inhibitors may perspective prove advantageous, especially in cases of platelet hyperreactivity that may be associated with MRP4 overexpression. Besides Ceefourin-1, other effective MRP4-inhibiting compounds have been recently published.²⁹ These were developed primarily for the reversal of drug resistance in MRP4-overexpressing cancer cells. Since tumors are often associated with thrombosis and aspirin has been recently recognized as a promising cancer-preventive agent probably based on anti-platelet-mediated effects,³⁰ one may speculate that MRP4 inhibitors may provide dual benefits in some tumor patients. Other compounds such as the phosphodiesterase-3 and MRP4 inhibitor cilostazol may affect platelet reactivity by a dual-action.^{10,16} Further studies are required to evaluate which MRP4 inhibitors may be best suitable for an *in vivo* application.

Disclosures

No conflicts of interests to disclose.

Contributions

RW and SG conducted and designed the main experiments and data analyses. RP, CT, EM, AB, MH, and AH provided additional analyses and methodological expertise. AG and MVT contributed to the study design, BHR and GJ designed and supervised the study. RW and GJ drafted and all authors edited the manuscript.

Acknowledgments

The authors thank Edita Kaliwe and Sarah Polster for their expert technical assistance. *Mrp4*-deficient (*Mrp4* (-/-)) mice were kindly provided by the late Dr. Gary D. Kruh, Cancer Center, University of Illinois, Chicago, USA.

Funding

This study was supported by grants from the Deutsche Forschungsgemeinschaft to GJ (DFG, JE 234/4-1) and to BHR (DFG, RA 1714/1-2). RW has received a doctoral scholarship

from the DZHK (Deutsches Zentrum für Herz-Kreislauf-Forschung e.V., grant 81X3400103). RP and AG receive support from Deutsche Forschungsgemeinschaft, grant/award number: 374031971-TRR 240. We also acknowledge support for the Article Processing Charge from the DFG and the Open Access Publication Fund of the University of Greifswald.

Data-sharing statement

All original data and protocols can be made available to other investigators upon request.

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