

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

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Online Supplementary Data

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This supplementary file includes:

1. Supplementary figures S1 –S4 and corresponding legends
2. Materials and Methods (detailed description)

Supplementary Figures

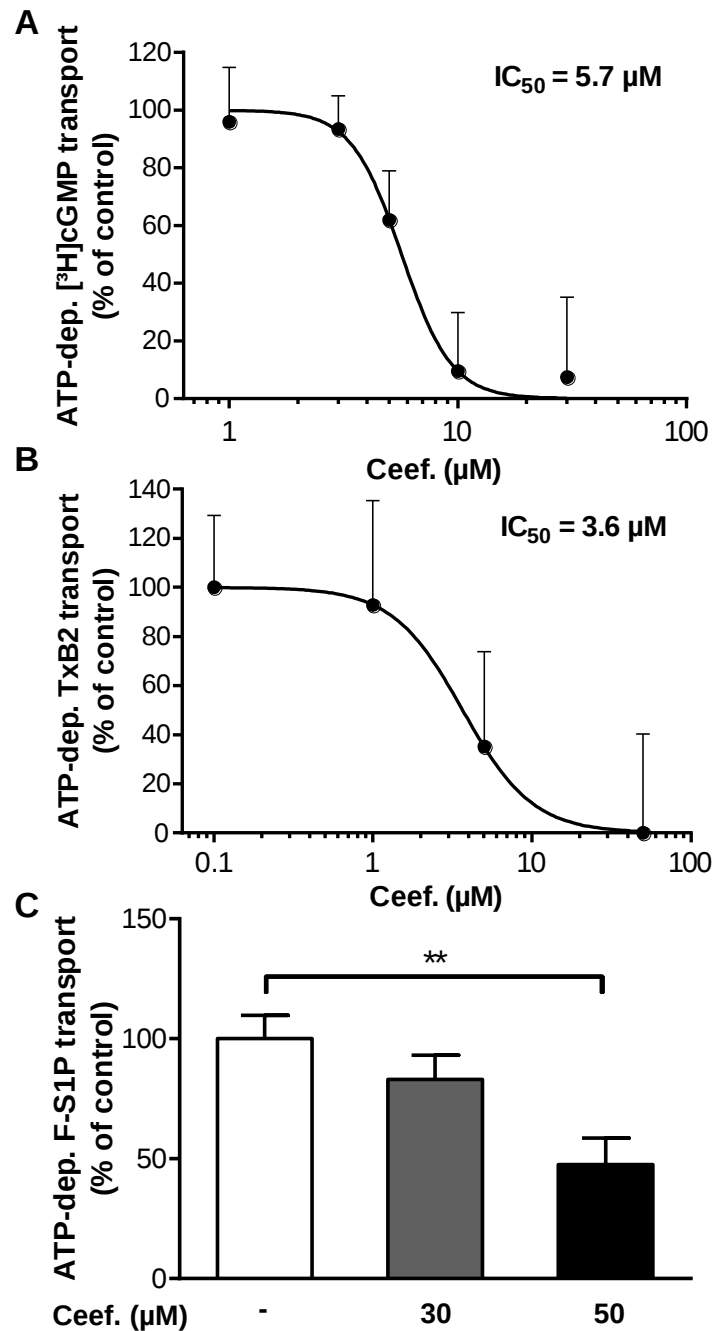


Figure S1: MRP4-mediated transport of several signaling compounds is inhibited by Ceefourin-1. Membrane vesicles from Sf9 cells recombinantly expressing human MRP4 were prepared as described before.¹ Vesicles were incubated with [³H]cGMP (2 μM) (A), thromboxane B2 (TxB2; 1 μM) (B), or fluorescein-labeled S1P (F-S1P, 1 μM) (C) in the presence of Ceefourin-1 (Ceef., in the indicated concentrations) and of 4 mM ATP or 5'-AMP for 10 min at 37°C. The vesicle-associated substrate was determined by rapid filtration using nitrocellulose filters (A) or centrifugation of the vesicles through Sephadex G-50 columns (B, C). [³H]cGMP and F-S1P amounts were determined using a liquid scintillation counter or a microplate fluorescence reader, respectively (for details see Vogt et al¹). TxB2 concentrations were determined by ELISA (see Fig. S2). ATP-dependent (ATP-dep.) transport rates were calculated by subtracting transport in the presence of 5'-AMP as a blank from transport in the presence of ATP and calculated as percentage of control. IC₅₀ values were calculated by nonlinear regression from the sigmoidal dose-response curve using GraphPad Prism 5.01 software. Values represent mean + SEM (n=6) **p<0.01 vs. control.

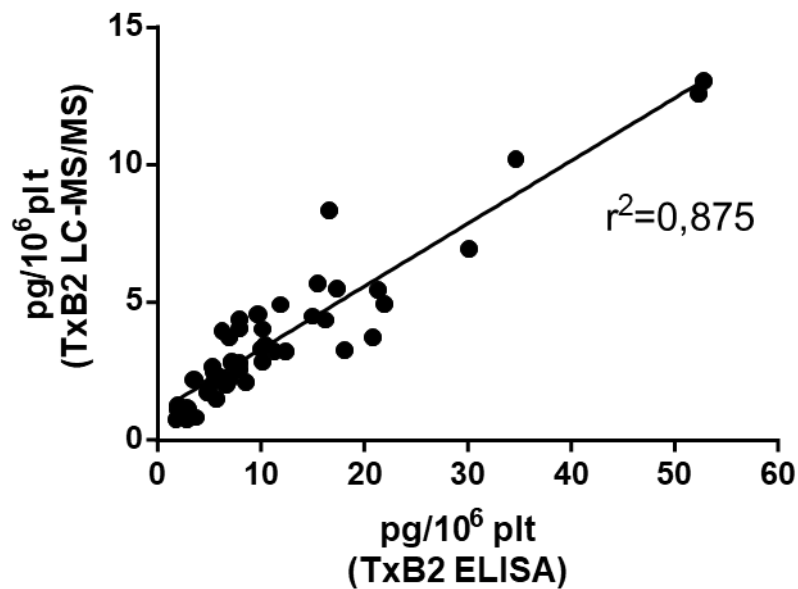


Figure S2: Comparison of TxB2 amounts in platelet supernatants determined by LC-MS/MS and ELISA. TxB2 concentrations were determined either by LC-MS/MS as described in the *Supplementary Methods* or using a commercially available TxB2 Elisa Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. Both methods showed comparable results for the decreased TxB2 release from human platelets after pre-treatment with Ceefourin-1 (Figure 1, main manuscript).

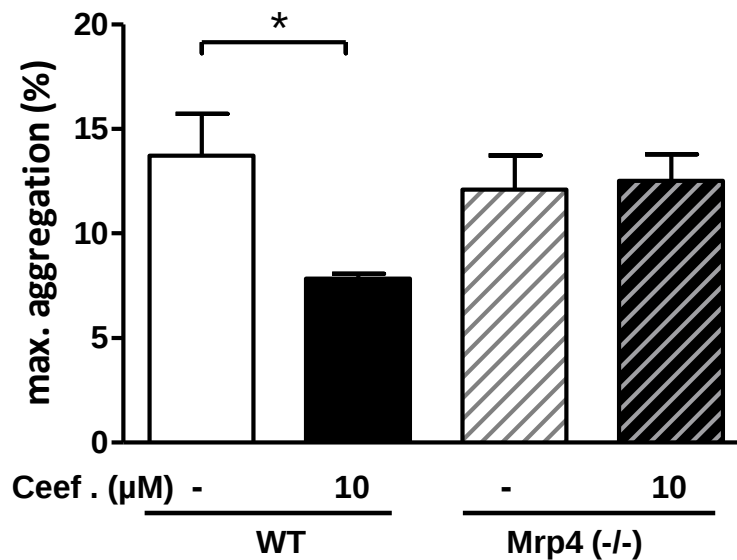


Figure S3: Effect of Ceefourin-1 on *ex vivo* aggregation of murine platelets stimulated with ADP. Blood of age- and sex-matched wild-type (WT) or Mrp4-deficient (Mrp4^{-/-}) mice was obtained by right ventricular heart puncture, diluted (1:2) with Tyrode's buffer and centrifuged to obtain platelet-rich plasma (PRP). Platelet aggregation was determined by light transmission aggregometry. Platelets were pre-treated with either only the solvent (control, (-)) or Ceefourin-1 (Ceef.; 10 μM) and then stimulated with ADP (10 μM). Aggregation curves were monitored and the maximal extent of aggregation (%) was calculated (Mean + SEM; n=4-5). Ceefourin-1 led to a significant reduction of the aggregation in WT platelets (*p<0.05). In Mrp4-deficient mice, Ceefourin-1 treatment resulted in no inhibition. However, in contrast to the results obtained with collagen (Figure 2C, main manuscript), the knock-out itself resulted only in a slight (here not significant) reduction of the aggregation. This discrepancy may be due to long-term adaptive mechanisms in the knock-out mice that compensate for the lack of Mrp4 when the platelets are only weakly activated.

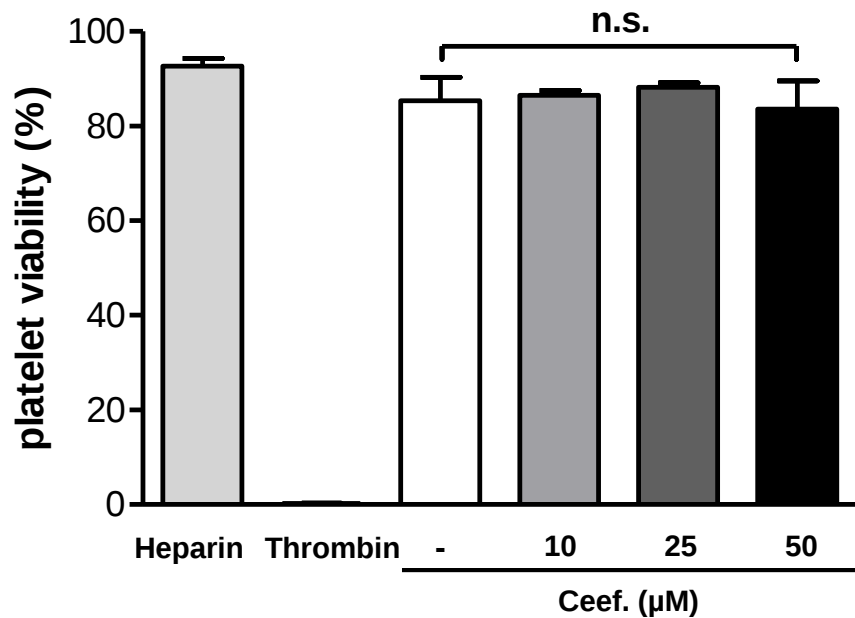


Figure S4: Effect of Ceefourin-1 on platelet viability. The accumulation of the fluorescent dye calcein in platelets was measured to indicate a loss of viability due to decreased membrane integrity and metabolic changes. Platelets were incubated with heparin (0.1 U/ml, negative control), thrombin (100 nM, positive control), DMSO (solvent control, (-)) or Ceefourin-1 (Ceef.; 10, 25 or 50 µM) prior to addition of the cell-permeable calcein-AM ester, which is intracellularly cleaved by esterases. The accumulation of calcein in platelets was analyzed by flow cytometry (488 nm excitation). The gate for the viable platelet population was established using the heparin-stabilized platelets. The thrombin control resulted in a loss of platelet viability associated with the activation. No significant loss of platelet viability was detected with Ceefourin-1 in concentrations of up to 50 µM compared to the solvent control (means + SEM; n= 6 measurements with PRP from 3 different donors).

Material and Methods (detailed description)

Antibodies and labeled compounds

For flow cytometric analyses: FITC-labeled human fibrinogen was purchased from Molecular Innovations, Peary Court, Novi, MI, USA, and R-phycoerythrin anti-human-CD62P and immunoglobulin G1 (IgG1)- κ -isotype matched control from BioLegend, San Diego, CA, USA. Phospho-specific rabbit polyclonal antibody against serine residue 157 of VASP and mouse monoclonal antibody against serine residue 239 were from Santa Cruz Biotechnology, Santa Cruz, CA, USA. The respective Alexa Fluor 488-conjugated secondary antibodies were obtained from Thermo Fisher Scientific, Waltham, MA, USA.

For flow chamber experiments: FITC-labeled mouse anti-human CD42a was purchased from BD Biosciences, Franklin Lakes, NJ, USA and DyLight 488-conjugated anti-mouse GPIIb β (X488) antibody from Emfret Analytics, Eibelstadt, Germany.

Light transmission aggregometry

Stock solutions of Ceefourin-1 (Abcam, Cambridge, UK) and cinaciguat (Cayman chemical, Ann Arbor, MI, USA) were prepared in DMSO and further diluted in Tyrode's buffer [NaCl 134, NaHCO₃ 12, KCl 2.9, CaCl₂ 2, NaH₂PO₄ 0.36, MgCl₂ 1, HEPES 5, glucose 5 (mM) and 1 mg/ml BSA (fatty acid-free), pH 7.4]. Aspirin (D, L-lysine acetylsalicylate glycine; Aspirin i.v.[®], Bayer, Leverkusen, Germany) was dissolved in water for injection. Platelet aggregation in PRP was induced by adding one of the following agents: collagen (5 μ g/ml), ADP (5 μ M) (both from Hart Biologicals, Hartlepool, UK), the activating peptide TFLLRN for the thrombin (protease-activated) receptor-1 (PAR1-AP; Biosynthan, Berlin, Germany) (30 μ M) or the thromboxane receptor agonist U46619 (Enzo Life Sciences, Lörrach, Germany) (2 μ M). Aggregation curves were monitored using an ATRACT-4004 light transmission aggregometer (LABiTec, Ahrensburg, Germany) and the maximal extent of aggregation (%) and the slope of the aggregation curve (%/min) were calculated.

Analysis of platelet thromboxane release

Platelets were pelleted from PRP, which was obtained by low-speed centrifugation (100 x g; 5 min at RT) and diluted in HEP-Puffer [NaCl 140, KCl 2.7, HEPES 3.8, EGTA 5 (mM), pH 7.4] with 0.5 μ M PGE₁, and resuspended in Tyrode's buffer supplemented with heparin (10 IU/ml) and PGE₁ (0.5 μ M) to prevent platelet activation during the washing process. After 10 min at 37°C, platelets were pelleted again and resuspended in Tyrode's buffer with apyrase (Sigma-Aldrich, Saint-Louis, MI, USA; 0.02 IU/ml). The platelet suspension was pre-incubated with either

Ceefourin-1 (50 μ M) or the respective solvent control (0.5 % DMSO) 15 min at 37°C. For platelet activation, collagen-related peptide (CRP-XL, kindly provided by R. Farndale, University of Cambridge, Cambridge, UK) or PAR1-AP were added. After additional 15 min incubation, platelets were spun down (1900 x g, 8 min) to separate platelet pellets and supernatants.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Concentrations of TxB2 in platelets and supernatants were determined by LC-MS/MS using the Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to an API 4000 mass spectrometer (AB Sciex, Darmstadt, Germany). Samples were supplemented with the respective internal standards (TxB2-d4; Cayman Chemical, Ann Arbor, MI, USA) and deproteinized with acetonitrile (80% v/v). For separation of TxB2 a Brownlee SPP RP-Amide column (PerkinElmer, Rodgau, Germany) and an isocratic elution with 70% acetonitrile/methanol (6:1 v/v)/30% formic acid (0.01%) was used (350 μ l/min flow; 40 °C). TxB2 and TxB2-d4 were quantified by mass transition of 369>168.7, 195 m/z and 373>172.7, 199 m/z, respectively.

Flow cytometric analyses

Fibrinogen binding to integrin α IIb β 3

PRP was diluted in HEPES-buffered saline (HBS) [NaCl 150, KCl 5, MgSO₄ 1, HEPES 10 (mM), pH 7.4] and incubated with Ceefourin-1 (10 - 50 μ M) or the respective concentration of the solvent (DMSO; control) for 15 min at RT. The samples were stimulated with either CRP-XL or ADP for 10 min, before FITC-labeled human fibrinogen and R-phycoerythrin anti-human-CD62P were added. After 20 min, samples were fixed in 0.2% formaldehyde. Flow cytometric acquisition of 5000 events was performed in a guava easyCyte™ device (EMD Millipore, Billerica, MA, USA). To set a negative control, an immunoglobulin G1 (IgG1)- κ -isotype matched control was used for the CD62P antibody and EGTA (10 mM) was included to prevent fibrinogen binding to integrin α IIb β 3.

VASP (vasodilator-stimulated phosphoprotein) phosphorylation

Washed platelets were resuspended in HBS buffer and incubated for 10 min 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 by addition of formaldehyde (2% final concentration), pelleted (8000 x g for 10 s at RT) and resuspended in 0.2% Triton-X100 (in PBS) for permeabilization. After 10 min, platelets were centrifuged again (2700 x g for 1 min at RT) and resuspended in PBS. Either the phospho-specific rabbit polyclonal antibody against serine residue 157 of VASP or the mouse monoclonal antibody against serine residue 239 was added (final concentration 3 μ g/ml) and

allowed to bind for 30 min at RT. After washing to remove unbound primary antibody, platelets were incubated with the respective Alexa Fluor 488-conjugated secondary antibodies for 20 min at 4°C in the dark. After two additional washing steps platelets were finally resuspended in PBS and flow cytometric acquisition of 5000 events was performed. Data were analyzed with InCyte™ Software (EMD Millipore, Billerica, MA, USA).

Platelet viability assay

To assess platelet viability, we used a protocol which is based on the intracellular accumulation of the fluorescent dye calcein. The assay was essentially performed as described by Ivetic et al.² In brief, PRP was diluted 1:10 in modified Tyrode's buffer [NaCl 137, NaHCO₃ 12, KCl 2.8, NaH₂PO₄ 0.4, MgCl₂ 1, HEPES 10, glucose 5.5 (mM), pH 7.4] and incubated with heparin (obtained from Rotexmedica, Trittau, Germany; 0.1 U/ml), thrombin (Enzo, New York, NY, USA; 100 nM), Ceefourin-1 (10 - 50 µM) or the respective concentration of the solvent (DMSO; control) for 15 min at 37°C. Subsequently, the samples were stained with calcein-AM (obtained from Merck, Darmstadt, Germany; 2 µg/ml). After incubation for 30 min at 37°C in the dark, PBS supplemented with EDTA (5 mM) was added 3:1 to each reaction to reduce any further platelet activation. The samples were analyzed by flow cytometry (488 nm excitation). The gate for the viable platelet population was established using the heparin-stabilized platelets and was applied for all other samples. The final data were analyzed with the InCyte™ Software.

Calcium measurements

Washed platelets were resuspended in calcium-free Tyrode's buffer supplemented heparin (10 IU/ml) and PGE₁ (0.5 µM) and incubated with Fura-2 AM (Biomol, Hamburg, Germany) (5 µM) for 45 min at 37°C in the dark. Platelets were pelleted and resuspended in calcium-free Tyrode's buffer (+ 0.5 µM PGE₁) and incubated for 10 min at 37°C in the dark. After a further washing step, platelets were resuspended in calcium-free Tyrode's buffer containing apyrase (0.02 IU/ml) and pre-incubated with the inhibitor for 10 min. The suspensions were either recalcified with CaCl₂ (2 mM) or calcium depleted by addition of EGTA (0.2 mM) and allowed to equilibrate before platelet agonists were added (modified protocol according to Ohlmann et al.³). Intracellular calcium was measured with a fluorescence spectrophotometer (LS55, Perkin Elmer, Waltham, MA, USA) with heated (37°C) quartz cuvettes, using the excitation wavelengths of 340 nm and 380 nm, while continuously monitoring the fluorescence emission at 510 nm for 3 min. After the measurements, Triton-X100 (0.1 %) was used to release all intracellular Fura-2, followed by the addition of EGTA (8 mM) to measure the fluorescent signals at nearly zero levels of free calcium.

Statistics

Data are given as mean $\pm$ standard error of the mean (SEM) and analyzed by one-way analysis of variance (ANOVA) or *t*-test and paired *t*-test (Figure 4B and C of the main manuscript, respectively) (GraphPad Prism 5.01 software, GraphPad, SanDiego, CA, USA). $P < 0.05$ was considered significant.

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