ARTICLE - Blood Transfusion

Inhibition of GPIb-α-mediated apoptosis signaling enables cold storage of platelets

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Supplementary methods

Study design

The aim of the present study was to investigate the efficacy of three compounds that have been reported to inhibit the apoptotic pathways (Figure 1) and to evaluate their impact on cold-stored platelet (CSP) function and half-life. The first compound, G04, inhibits upon binding the RhoA GTPase protein, a molecular switcher, which regulates the transduction of the intracellular apoptotic signal induced by GPIb-alpha clustering on the platelet membrane.[1] The second one, forskolin, upregulates adenylyl cyclase (AC), which in turn enhances the formation of cAMP. The latter activates the protein kinase A (PKA), which inhibits the apoptotic signal by phosphorylating several targets like the pro-apoptotic protein Bad.[2] The third compound, caspase-9 inhibitor, prevents the autocatalytic cleavage and activation of caspase-9, which is one of the key final steps of the apoptotic signal.[3]

Preparation of apheresis platelet concentrates

Platelet concentrates (PCs) were collected using the apheresis device TRIMAAccel 7.0 (TERUMO BCT, Munich, Germany) from healthy volunteers according to the German guidelines for hemotherapy after obtaining written consensus. Briefly, 166 mL platelet rich plasma (PRP) were collected and at the end of the separation process were resuspended in 271 mL additive solution (PASIII, Machropharm, Germany). After collection, PCs were allowed to rest for 1 hour (h) at room temperature (RT). Next, PC from one donor was split under sterile condition in different pediatric oxygen permeable bags (20 mL each bag, Fresenius Kabi AG, Bad Homburg, Germany) and each inhibitor was added in the corresponding bag. The optimal concentrations for G04 and forskolin were determined performing titration tests (Supplementary figure 1). The following concentrations were finally used in the current study: 150 µM G04 (RhoA Inhibitor, Millipore Corp., Darmstadt, Germany) and 0.75 µM forskolin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). 40 µM caspase-9 inhibitor (Z-LEHD-fmk, BD Biosciences, San Jose, USA) was used as final concentration, as previously described to treat platelets.[4] All inhibitors were diluted in dimethyl sulfoxide (DMSO). To exclude any effect of the vehicle (DMSO), we performed pre-tests to analyzed platelet apoptosis (Supplementary figure 2) and functionality (Supplementary figure 3), using PCs incubated at 4°C with 2% DMSO (the highest concentration of DMSO used to dilute the inhibitors). All PCs were stored at 4 °C under constant agitation on a standard platelet agitator (Heidolph, Frankfurt, Germany) for 10 days and samples were collected after 1, 4, 7 and 10 days of storage for further analyses.

Determination of apoptosis

Assessment of the mitochondrial inner transmembrane potential

To detect changes in the mitochondrial inner transmembrane potential, the tetramethylrhodamine ethyl ester (TMRE) assay kit (Abcam, Cambridge, UK) was used as previously described, with minor modifications.[5] Briefly, CSPs (300,000 cells/µL) were stained with 10 mM TMRE (30 minutes [min] at RT) and directly measured by flow cytometry (FC) (Navios, Beckman-Coulter, Krefeld, Germany). As positive control, cells were incubated with the uncoupler of mitochondrial oxidative phosphorylation carbonyl cyanide 4-trifluoromethoxy phenylhydrazone (FCCP, 10 mM, 30 min at 37°C) which induces a complete depolarization of platelet mitochondrial potential. Data were reported as mean fluorescence intensity (MFI) of TMRE signal.

Detection of phosphatidylserine *externalization*

The externalization of phosphatidylserine (PS) was determined upon Annexin-V staining, as previously described with minor modifications.[5] Briefly, CSPs (300,000 cells/µL) were stained with 1 µL Annexin V-APC (Thermo Fisher Scientific, Waltham, USA) and CD41-PE-Cy5 (Beckman Coulter) in TRIS (Trisaminomethane) buffer for 20 min at RT and measured by FC. CD41-PC5 positive cells were analyzed for Annexin V-APC binding reflecting surface externalization of PS. Platelets freshly isolated from healthy donors were incubated with 10 mM ionomycin (Abcam, Cambridge, UK) and used as positive control.

Platelet functionality

Platelet aggregation

Light transmission platelet aggregation assay was performed using a 4-channel-aggregometer (LABiTec, LAbor BioMedical Technologies, Ahrensburg, Germany). Platelets (300,000 cells/µL) were incubated with 20 µM thrombin receptor-activating peptide-6 (TRAP-6, Hart Biologicals, Hartlepool, UK), 1.0 mg/mL ristocetin (HART Biologicals, Hartlepool, United Kingdom) or NaCl (Braun, Melsungen, Germany) as control. The maximal aggregation and agglutination were registered during 6 min of measurement at 37 ºC. Fresh platelets were used as positive control.

Platelet adhesion

The adhesion ability of CSPs was assessed as previously described with minor modifications.[5] In brief, coverslips (Corning, New York, USA) were coated overnight with 100

mg/mL of fibrinogen (Sigma Aldrich, Munich, Germany) or 5% human serum albumin (Grifols, Munich, Germany). Next, CSPs ($1x10^8$ cells/mL) were allowed to seed on coverslips for 1 h at RT in the presence of 10 µM TRAP-6. The adherent cells were fixed with 2% paraformaldehyde (Morphisto, Frankfurt, Germany) for 20 min at RT. Images were captured (x100, Olympus IX73, Tokyo, Japan) and analyzed with the CellSens Standard software (Olympus). The total number of adherent cells as well as the percentage of the different platelet phenotypes (Type 1, resting cells with discoid shape; type 2, cells with filopodia; type 3, cells with lamellipodia and type 4, fully spreaded platelets) were quantified from 6 different microscopic fields per coverslip, respectively. Immunofluorescences were performed to stain the adherent cells using the primary monoclonal mouse antibody against human GPIIb/IIIa complex overnight at 4°C (1:100, Gi5, Enzo Life Sciences, Farmingdale, USA) and followed by incubation with the secondary monoclonal antibody goat anti mouse IgG for 1 h at RT (1:400, AlexaFluor™ 488, Invitrogen, Eugene, USA).

Clot retraction assay

The clot retraction assay was performed to investigate platelet functions in term of interaction between fibrin outside the cells and the cytoskeleton of the platelets. In brief, 300 µL of undiluted CSPs suspension were incubated with 0.1 M $CaCl_2$ (Sigma Merck, Darmstadt, Germany) as well as thrombin 10 U/mL (Roche, Mannheim, Germany) at RT and pictures were taken after 1 h. PRP from healthy donors were used as control. Finally, pictures were analyzed using the ImageJ software and clot surfaces were calculated as percentage of retraction area compared to the total area, as previously described.[6]

Thromboelastography

Thromboelastography was performed to investigate the kinetic of clot formation and stability of formed clots using the ClotPro analyzer (Haemonetics, Munich, Germany), according to the manufacturer's instructions. The clot formation of platelets-depleted full blood samples from healthy donors spiked-in with CSPs was analyzed. In brief, citrated whole blood from healthy donors was centrifuged (20 min, 120 g, without brakes) and PRP was collected. The latter was additionally centrifuged (10 min, 2000 g) and the PPP was collected and used to dilute the CSPs in order to reach the same volume and cell count of PRP obtained upon the first centrifugation step. Finally, CSPs were gently added back to reconstitute whole blood samples and analyzed (Supplementary figure 4). The following parameters were measured in the extrinsic test: maximum clot firmness, which reflects the absolute strength of the fibrin and platelets clot; and maximum lysis, which indicates the degree of fibrinolysis relative to percentage clot firmness lost.[7] Untreated whole blood from the same donors was used as control.

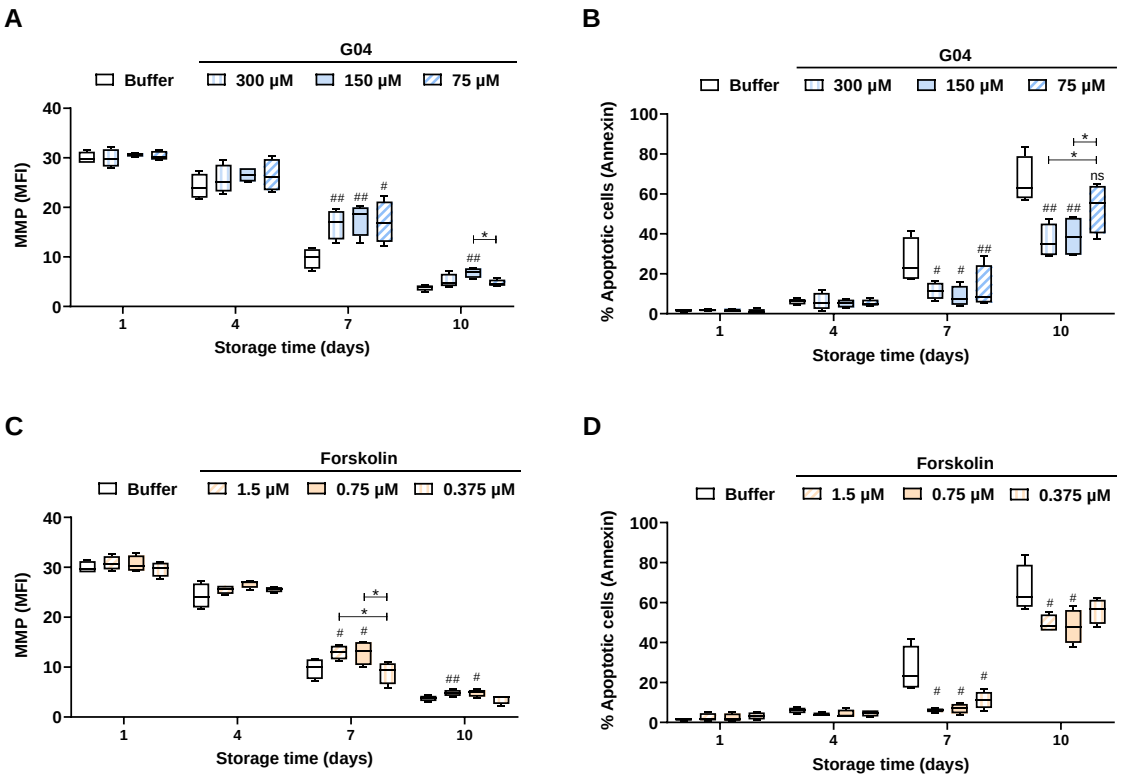
In vivo studies

To determine the survival of human CSPs, we used the NSG [NOD (Non-obese diabetic) Scid Gamma)] mouse model (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, stock No. complexes, 005557) purchased from the Jackson Laboratories (Charles River, Research Models and Services, Sulzfeld, Germany). The experiment procedure was performed as previously described with minor modifications.[5] Sex- and age-matched (10-12 weeks) animals were used in this study. Platelets (4x10^9 cells/mL, final volume 200 µL) were injected into the lateral tail vein and after 30 min a blood sample was collected by tail vein punctuation to determine the baseline of circulating human platelets (100%). The survival of human platelets in the mouse bloodstream was measured by taking periodical murine blood after 1, 2, 5 and 24 h. Samples were prepared immediately after collection using a commercially available fixation kit (PerFix-nc Kit, Beckman Coulter, Brea, CA, USA). Briefly, 20 µL of murine blood were collected into 30 µL of acid-citrate-dextrose (ACD-A; BD Bioscience, San Diego, CA, USA), fixed with fixation buffer (1:10) for 15 min at RT and red blood cells were lysed using 100 µL of lysis buffer. Next, samples were stained with anti-human CD41-PE-Cy5 (Beckman Coulter) and anti-mouse CD41-FITC (BD Bioscience) for 30 min at RT, resuspended in buffer (1:10) and measured by FC. Platelets were distinguished from other cells by means of size (forward scatter, FSC), granularity (side scatter, SSC) and positivity for human CD41. As control, platelets from PCs stored at RT for 24 h were injected into the mouse circulation (Supplementary figure 5).

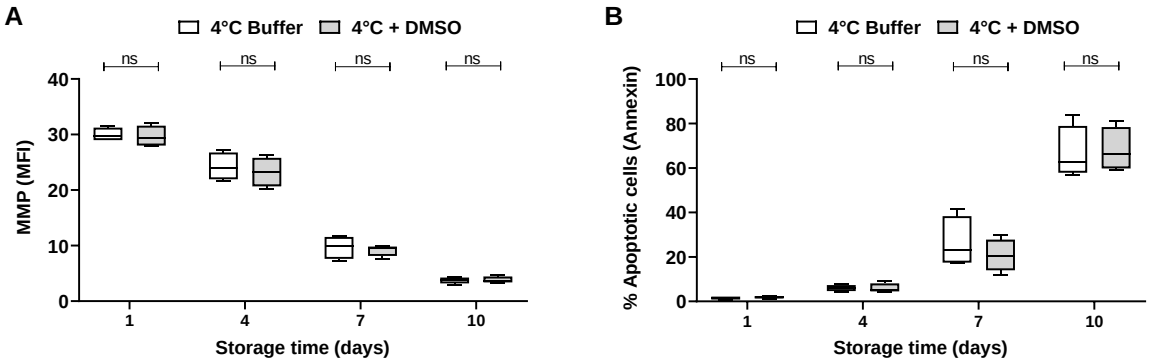
Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.4.1 (GraphPad Software, La Jolla, USA). A paired t-test was used to analyze normally distributed results. Non-parametric tests were used when data failed to follow a normal distribution as assessed by the D'Agostino and Pearson omnibus normality test. Group comparison was performed using the Wilcoxon rank-sum test and the Fisher exact test with categorical variables. $P<0.05$ was considered statistically significant.

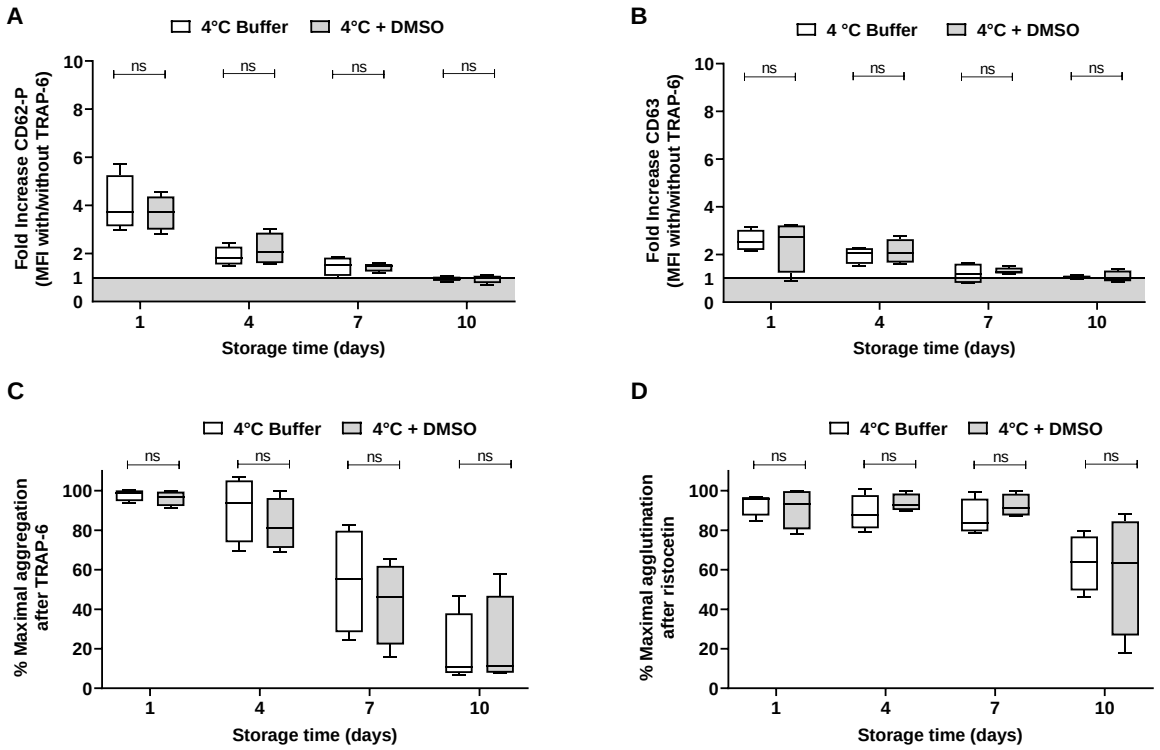
Supplementary Figures



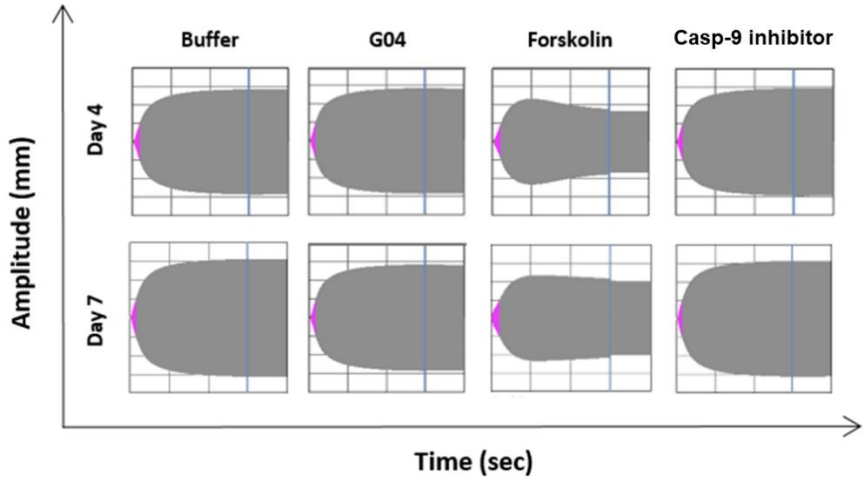
Supplementary Figure 1: Titration of G04 and forskolin.
After 1, 4, 7 and 10 days of storage the mitochondrial membrane potential (MMP; A, C) and Annexin (B, D) were measured by flow cytometry. Platelet concentrates were stored at 4°C in buffer or with 300 µM, 150 µM and 75 µM of G04 (A and B) or 1.5 µM, 0.75 µM and 0.375 µM of forskolin (C and D), respectively. Data are shown as box and whiskers±Standard Error of Mean; * or # p<0.05; ** or ## p<0.01, n=4. The symbol # indicates comparisons between buffer and each inhibitor. When not indicated the data were not significant.



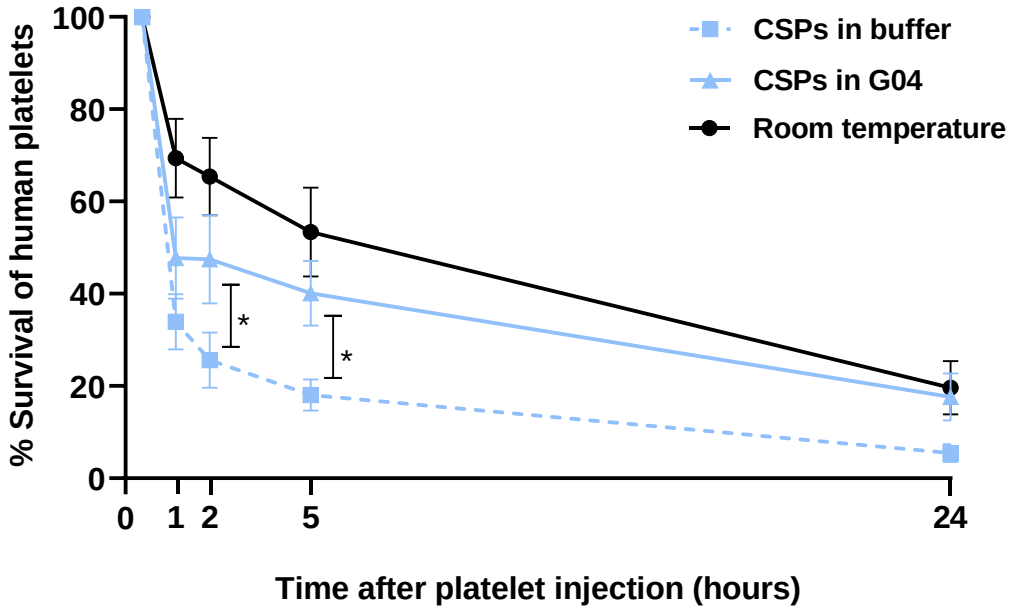
Supplementary Figure 2: Cold-induced apoptosis inhibition in the presence of DMSO.
After 1, 4, 7 and 10 days of storage the mitochondrial membrane potential (MMP; A) and Annexin (B) were measured by flow cytometry. Platelet concentrates were stored at 4°C in buffer (white symbols) or with DMSO (grey symbols). Data are shown as box and whiskers±Standard Error of Mean. ns: not significant, n=4.



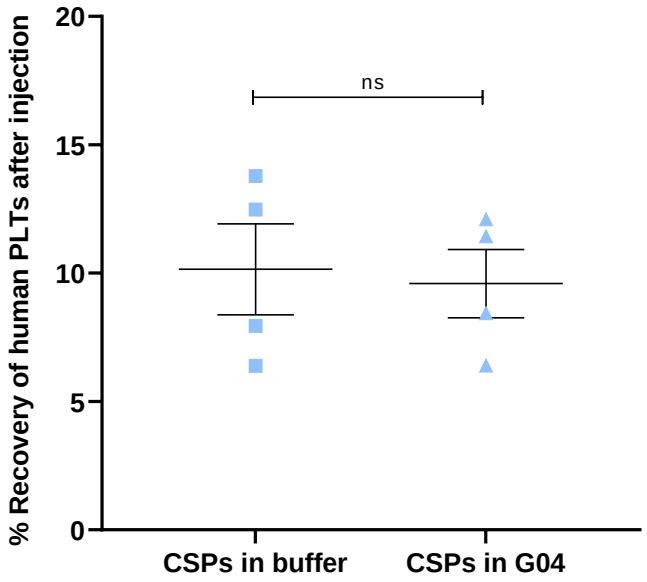
Supplementary Figure 3: Functionality of cold-stored platelet incubated with DMSO. Platelet concentrates were stored at 4°C in buffer (white symbols) or with DMSO (grey symbols) for 1, 4, 7 and 10 days. The expression of CD62-P (A) and CD63 (B), in response to TRAP-6 (10 µM), was determine by flow cytometry. The maximal aggregation ability (C) as well as the maximal agglutination (D) were measured upon stimulation with the inductors TRAP-6 (20 µM) and ristocetin (1 mg/mL), respectively. Data are shown as box and whiskers±Standard Error of Mean. ns: not significant, n=4.



Supplementary Figure 4: Representative curves of thromboelastography of cold-stored platelets.
The ability of cold-stored platelets to form clots was analysed performing a thromboelastography assay (extrinsic test, Figure 6). Representative curves, obtained measuring each inhibitor (G04, forskolin and caspase-9 inhibitor) after 4 and 7 days of storage are reported, respectively. Y-axis: Amplitude (millimetres, mm), x-axis: time (seconds, sec).



Supplementary Figure 5: Survival of platelet concentrates stored at 4°C with and without the apoptosis inhibitor G04 or at room temperature.
Cold-stored platelets (CSPs) stored for 7 days in buffer (full squares and dashed blue line) or in the presence of G04 (RhoA inhibitor; full tringles and continues blue line) as well as platelets stored for 24 h at room temperature (full circles and black line) were administered into the mouse circulation via the lateral tail vein. Survival of human platelets in the mouse circulation was analyzed by flow cytometry by collecting murine blood 1, 2, 5 and 24 hours post injection. Data are shown as mean±Standard Error of Mean. *$p<0.05$; if not indicated the data were not significant, n=4.



Supplementary Figure 6: Recovery of platelet concentrates stored at 4°C with and without the apoptosis inhibitor G04.
Cold-stored platelets (CSPs) stored for 7 days in buffer (full blue squares) or in the presence of G04 (RhoA inhibitor; full blue tringles) were administered into the mouse circulation via the lateral tail vein. Recovery of human platelets in the mouse circulation was analyzed by flow cytometry by collecting murine blood 30 minutes after injection. Data are shown as mean±Standard Error of Mean. ns: not significant, n=4.

	Day 1	Day 4	Day 7	Day 10
Apoptosis	⇔ ⇔ ⇔	⇔ ⇔ ⇔	↓ ↓ ↓	↓ ↓ ↓
Activation CD62	⇔ ↓ ⇔	⇔ ↓ ⇔	⇔ ⇔ ⇔	↑ ⇔ ⇔
Activation CD63	↑ ⇔ ⇔	⇔ ↓ ⇔	↑ ↓ ↓	↑ ⇔ ⇔
Aggregation (TRAP-6)	⇔ ↓ ⇔	⇔ ↓ ⇔	⇔ ↓ ⇔	↑ ⇔ ⇔
Agglutination (Ristocetin)	⇔ ⇔ ⇔	⇔ ⇔ ⇔	⇔ ↓ ⇔	↑ ⇔ ⇔
Adhesion	x	↑ ⇔ ⇔	⇔ ⇔ ⇔	x
Clot retraction	x	⇔ ↓ ⇔	⇔ ↓ ⇔	x
Thromboelastography (MCF)	⇔ ⇔ ⇔	⇔ ↓ ⇔	⇔ ⇔ ⇔	⇔ ⇔ ⇔
Thromboelastography (ML)	⇔ ↓ ⇔	⇔ ↓ ⇔	⇔ ↓ ⇔	⇔ ↓ ⇔
Survival *in vivo*	x	x	↑	x

G04
Forskolin
Casp-9 inhibitor

Supplementary Table 1: Summary of the efficacy and impact of apoptosis inhibitors on platelet functionality and survival.

All data were compared to buffer at the indicated storage time point.
Symbols description, Upwards arrows: increased functionality/survival compare to samples incubated with buffer; Downwards arrows: decreased functionality/survival compared to samples incubated with buffer and Left/right arrows: comparable functionality/survival in comparison to samples incubated with buffer.
Colour description, blue: G04; orange: forskolin and green: caspase-9 inhibitor.
Abbreviations, TRAP-6: thrombin receptor-activating peptide-6; MCF: maximum clot firmness; ML: maximum lysis.

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