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

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Running head: Cold-induced apoptosis inhibition in platelet concentrates

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I.M., K.A., S. N.-H., E.S. and T.B. designed the study. I.M., L.P., Y.T., C-T.M., and A.W. performed the experiments. I.M., L.P. and T.B. analyzed the data and interpreted the results. I.M and T.B. wrote the manuscript. All authors read and approved the manuscript.

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Abstract

Cold storage of platelets has been suggested as an alternative approach to reduce the risk of bacterial contamination and to improve the cell quality as well as functionality compared to room temperature storage. However, cold-stored platelets (CSPs) are rapidly cleared from the circulation. Among several possible mechanisms, apoptosis has been recently proposed to be responsible for the short half-life of refrigerated platelets. In the present study, we investigated the impact of apoptosis inhibition on the hemostatic functions and survival of CSPs. We found that blocking the transduction of the apoptotic signal induced by glycoprotein Ib (GPIb)-alpha clustering or the activation of caspase 9 does not impair CSPs functionality. In fact, the inhibition of GPIb-alpha clustering mediated-apoptotic signal by a RhoA inhibitor better conserved delta granule release, platelet aggregation, adhesion and the ability to form stable clots, compared to untreated CSPs. In contrast, upregulation of the protein kinase A caused a drastic impairment of platelet functions and whole blood clot stability. More importantly, we observed a significant improvement of the half-life of CSPs upon inhibition of the intracellular signal induced by GPIb-alpha clustering. In conclusion, our study provides novel insights on the in vitro hemostatic functions and half-life of CSPs upon inhibition of the intracellular cold-induced apoptotic pathway. Our data suggest that the combination of cold storage and apoptosis inhibition might be a promising strategy to prolong the storage time without impairing hemostatic functions or survival of refrigerated platelets.
Introduction

Transfusion of platelet concentrates (PCs) is an essential medical approach to treat bleeding in thrombocytopenic patients.\(^1\) Currently, PCs are stored at room temperature (RT, 22-24°C). At this storage condition PCs might be associated with increased risk of bacterial contamination\(^2\), post-transfusion septic reaction\(^3-4\) and platelet storage lesions (PSLs).\(^5\) Therefore, the storage time of PCs is restricted to 4-7 days, depending on the national guidelines.\(^1\) Nevertheless, the risk of transmission of bacterial infection still remains high.\(^2,6\) Moreover, recent clinical studies reported on detrimental effects in patients who received RT-stored PCs.\(^7,8\) In this context, alternative storage conditions like cold storage have been considered. In fact, cold storage might reduce the risk of bacterial contaminations, protect cells from storage lesions and extend products’ shelf-life.\(^9,10\) However, clinical applications of cold-stored platelets (CSPs), even if characterized by better functionality\(^9-14\), have been abandoned due to concerns regarding poor recovery and survival compared to RT.\(^15\) In fact, as reported in a recent radiolabeling study, the survival of autologous CSPs upon 5 days of storage was significantly lower (around 20%) compared to platelets stored for 7 days at RT (around 50%).\(^16\) However, this drawback of CSPs might be compensated by better functionality.

Several groups have investigated potential mechanisms underlying the observed short half-life of CSPs. One of the first proposed mechanisms was the desialylation of glycoproteins (GPs).\(^17\) However, a clinical study showed that reconstitution of sialylation does not prevent the accelerated clearance of CSPs.\(^18\) This indicates that alternative mechanisms, which do not require desialylation, trigger the fast elimination of refrigerated platelets. Interestingly, we and others detected increased apoptosis levels in platelets upon cold storage compared to RT suggesting that the apoptotic pathway might contribute to the reduced half-life of CSPs.\(^9,16,19\) In fact, cold storage was shown to trigger clustering of GPIb-alpha\(^20\) on the platelet surface leading to apoptosis.\(^19,21\) Interestingly, it has been reported that the presence of an apoptosis inhibitor (p38 inhibitor) reduces the expression of apoptotic markers, like Bax and Bak, in pathogen-inactivated PCs stored at RT but it did not improve platelet’s survival in vivo.\(^22\)

In the present work, we aimed to verify the efficacy of three compounds that have been shown to inhibit the apoptotic intracellular signal at different levels by targeting three key regulatory proteins (Figure 1 and supplementary methods). Our hypothesis was that all compounds could reduce cold-induced apoptosis. Nevertheless, since the proteins targeted by the inhibitors are involved in essential mechanisms of platelet functionality we thought to investigate potential
desire/undesired effects of each inhibitor on different cell functions in order to screen them and select the most promising one to perform \textit{in vivo} studies.

\textbf{Methods}

\textit{Preparation of platelet concentrates}

PCs were collected using the apheresis device TRIMAAcel 7.0 (TERUMO BCT, Munich, Germany). Briefly, 166 mL platelet rich plasma (PRP) were collected and resuspended in 271 mL additive solution (PASIII, Machropharm, Germany). Next, three inhibitors were added to inhibit the apoptotic signals. The first compound (G04) targets the RhoA GTPase, which has been shown to play an essential role in the transduction of the outside/inside signal downstream of GPIb-alpha.\textsuperscript{23} Furthermore, we tested a compound (forskolin), which upregulates the protein kinase A (PKA), via adenylate cyclase (AC), that is known to control the apoptotic pathway by inhibiting the pro-apoptotic protein Bad upon phosphorylation (Figure 1).\textsuperscript{24} The last tested compound (Z-LEHD-fmk) prevents the autocatalytic cleavage of caspase-9 that is necessary to begin the caspase cascade (Figure 1).\textsuperscript{25} The following final concentrations were used: 150 µM G04 (RhoA Inhibitor, Millipore Corp., Darmstadt, Germany), 0.75 µM Forskolin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and 40 µM caspase-9 inhibitor\textsuperscript{26} (Z-LEHD-fmk, BD Biosciences, San Jose, USA). Apoptosis inhibitors were added to different apheresis bags and stored at 4°C under agitation. See supplementary methods for further details.

\textit{Detection of platelet apoptosis}

To detect changes in the mitochondrial membrane potential (MMP), the tetramethylrhodamine ethyl ester (TMRE) assay kit (Abcam, Cambridge, UK) was used and the externalization of phosphatidylserine (PS) was determined using Annexin-V (Immunotools, Friesoyhte, Germany) staining. See supplementary methods for further information.

\textit{Assessment of platelet functionality}

To analyze platelet activation, CSPs were stimulated with thrombin receptor-activating peptide-6 (TRAP-6, 10 µM, HART Biologicals, Hartlepool, United Kingdom) and incubated with CD62-P (CLB-Thromb/6, Beckman Coulter, Krefeld, Germany) or CD63 (CLB-Gran/12, Beckman Coulter) and measured by flow cytometry (FC) (Navios, Beckman-Coulter, Krefeld, Germany).
Light transmission platelet aggregation assay was performed using a 4-channel-aggregometer (LABiTec, LAbor BioMedical Technologies, Ahrensburg, Germany) to investigate the aggregation ability in response to 20 µM TRAP-6, 1.0 mg/mL ristocetin (HART Biologicals, Hartlepool, United Kingdom) or NaCl (Braun, Melsungen, Germany). Next, the adhesion ability of CSPs was assessed as previously described using coverslips (Corning, New York, USA) coated with 100 mg/mL fibrinogen (Sigma Aldrich, Munich, Germany) or 5% human serum albumin (Grifols, Berlin, Germany). CSPs were allowed to seed on coverslips in the presence of 10 µM TRAP-6. Platelet clot retraction was assessed upon incubation with 7.4 µM CaCl₂ (Sigma Aldrich, Darmstadt, Germany) and thrombin 10 U/mL (Roche, Mannheim, Germany). Thromboelastography was performed using the ClotPro analyzer (Haemonetics, Munich, Germany) according to the manufactory instructions. See supplementary methods for further information concerning each assay.

**In vivo studies**

To determine the survival and recovery of human CSPs, we used an NSG [NOD (Non-obese diabetic) Scid Gamma)] mouse model. The experiment procedure was performed as previously described with minor modifications. See supplementary methods and supplementary figures 5 and 6 for additional information.

**Ethics and statistical analysis**

All studies involving human subjects were approved by the ethics committees of the University Hospital of Tuebingen. Animal studies were approved by the state animal ethics committees of Baden-Wuerttemberg. Statistical analyses were performed using GraphPad Prism 9.4.1 (GraphPad Software, La Jolla, USA). See supplementary methods for additional details.

**Results**

**Inhibition of cold-induced platelet apoptosis**

In order to verify the efficacy of the apoptosis inhibitors during cold storage, two of the key steps of the apoptotic pathway were analyzed, the reduction of MMP and the PS externalization. We found that CSPs started showing apoptotic phenotype from storage day 4, as suggested by the significant decrease of MMP compared to day 1 (MMP mean fluorescence intensity [MFI])
mean±standard error mean [SEM]: buffer day 1 vs. day 4, 30.00±0.61 vs. 24.33±1.26, p=0.0076; Figure 2A-C, respectively). This effect was time-dependent (MMP MFI mean±SEM: buffer day 4 vs. day 7, 24.33±1.26 vs. 9.74±1.05, p=0.0001; buffer day 7 vs. day 10, 9.74±1.05 vs. 3.76±0.29, p=0.0081; Figure 2A-C, respectively). The inhibition of RhoA (G04, Figure 2A, D), activation of PKA (Forskolin, Figure 2B, E) and downregulation of the autocalytic cleavage of caspase-9 (Figure 2C, F) induced a significant reduction of the apoptotic signal. The mitochondrial integrity was better maintained on day 7 and 10, as indicated by the significant higher levels of MMP, in comparison to untreated cells (MMP MFI mean±SEM: G04 vs. buffer day 7, 17.65±1.64 vs. 9.74±1.05, p=0.0091; day 10, 6.81±0.53 vs. 3.76±0.29, p=0.0080; forskolin vs. buffer day 7, 12.92±1.23 vs. 9.73±1.05, p=0.0434; day 10, 4.81±0.37 vs. 3.76±0.29, p=0.0404; caspase-9 inhibitor vs. buffer day 7, 12.45±0.83 vs. 9.74±1.05, p=0.0384; day 10, 5.08±0.61 vs. 3.76±0.29, p=0.0477; Figure 2A-C, respectively).

Similarly, treatment of CSPs with apoptosis inhibitors revealed a significant reduction of the percentage of cells exposing PS starting from storage day 7 compared to cells stored in buffer (% Apoptotic cells, mean±SEM: G04 vs. buffer day 7, 9±3% vs. 26±6%, p=0.0218; day 10, 39±5% vs. 67±6%, p=0.0023; forskolin vs. buffer day 7, 7±1% vs. 26±6%, p=0.0084; day 10, 48±4% vs. 67±6%, p=0.0319; caspase-9 inhibitor vs. buffer day 7, 10±3% vs. 26±6%, p=0.0105; day 10, 48±5% vs. 67±6%, p=0.0371; Figure 2D-F, respectively). Of note, after 10 days of storage all treated-CSPs showed increased PS externalization, compared to storage day 7. Nevertheless, the percentage of cells exposing PS on day 10 was significantly lower in the presence of the inhibitors than cells stored in buffer (Figure 2D-F).

These results indicate that pre-treatment of PCs with apoptosis inhibitors can prevent the cold-induced apoptosis.

The inhibition of GPIb-alpha clustering signal better maintains delta granule release and preserves platelet aggregation and agglutination in cold-stored platelets

The impact of apoptosis inhibition on platelet activation and aggregation was analyzed using FC and aggregometry, respectively. First, the release of alpha (CD62-P) and delta (CD63) granules content was determined upon TRAP-6 stimulation (Figure 3). The inhibition of either GPIb-alpha clustering signal, induced by G04 incubation, or caspase-9 showed unchanged alpha granule secretion compared to untreated-CSPs (Figure 3A, C). On the contrary, the incubation with the PKA agonist induced a significant reduction of the alpha granule secretion, already after 1 day of
cold storage (Fold increase [FI] CD62-P mean±SEM: forskolin vs. buffer day 1, 2.03±0.24 vs. 4.18±0.74, p=0.0487; day 4, 0.91±0.16 vs. 2.25±0.39, p=0.0229; Figure 3B).

Upon apoptosis inhibition with G04 a significant increase in the release of delta granule content was detected, in a time-dependent manner, compared to untreated-CSPs (FI CD63 mean±SEM: G04 vs. buffer day 1, 4.54±0.79 vs. 2.19±0.22, p=0.0394; day 7, 2.70±0.46 vs. 1.53±0.04, p=0.0466; day 10, 1.90±0.20 vs. 1.19±0.17, p=0.0133; Figure 3D). Upregulation of PKA as well as caspase-9 inhibition significantly impaired the TRAP-6-induced delta granule secretion on storage day 4 and 7 (FI CD63 mean±SEM: forskolin vs. buffer day 4, 1.38±0.20 vs. 2.04±0.18, p=0.0333; day 7, 1.04±0.01 vs. 1.53±0.04, p=0.0005; caspase-9 inhibitor vs. buffer day 7, 1.14±0.10 vs. 1.53±0.04, p=0.0058; Figure 3E and F, respectively).

Furthermore, we found that the inhibition of apoptosis induced by both G04 and caspase-9 show similar aggregation and agglutination ability upon stimulation with TRAP-6 and ristocetin compared to untreated cells (Figure 4A, D and 4C, F, respectively). Of note, enhanced aggregation and agglutination were detected after 10 days of cold storage only in the presence of the inhibitor G04 compared to cells stored in buffer (% Maximal aggregation mean±SEM: G04 vs. buffer day 10, after TRAP-6, 53±9% vs. 19±9%, p=0.0192; after ristocetin, 83±4% vs. 63±7%, p=0.0108; Figure 4A, D, respectively). Similarly to the activation results (Figure 3B, E), upregulation of PKA caused a drastic reduction of the aggregation ability in response to TRAP-6 in comparison to untreated-CSPs, already after 1 day of storage, with a time-dependent trend (% Maximal aggregation mean±SEM: forskolin vs. buffer, day 1, 90±3% vs. 98±2%, p=0.0311; day 4, 15±5% vs. 91±8%, p<0.0001; day 7, 13±1% vs. 54±13%, p=0.0275; Figure 4B, respectively). The same undesired effect was detected upon ristocetin stimulation but only on storage day 7 (% Maximal agglutination mean±SEM: forskolin vs. buffer, 58±6% vs. 86±5%, p=0.0045, Figure 4E).

Taking together, these data indicate that inhibition of GPIb-alpha clustering-mediated signal, induced by G04 incubation, better maintains delta granule release of CSPs without affecting the alpha granule secretion as well as the aggregation and agglutination abilities.

**The impact of apoptosis inhibition on cold-stored platelet adhesion**

Analyzing the adhesion ability of CSPs to fibrinogen we found that the total number of adherent cells detected upon inhibition of RhoA was significantly higher in comparison to untreated-CSPs
on storage day 4 (Number of adherent cells/field mean±SEM: G04 vs. buffer, day 4, 93±17 vs. 49±13, p=0.0493, Figure 5A). Similar adhesion ability was observed upon upregulation of PKA and inhibition of caspase-9 compared to cells stored in buffer, after 4 and 7 days of cold storage (Figure 5A-B).

Platelet adhesion is a multistep process, which leads to a complex reorganization of the cytoskeleton resulting in the formation of filopodia/lamellipodia ending with a complete spreading of the cells. Therefore, we analyzed the morphology of CSPs by quantifying the percentage of the different spreading patterns (type 1: resting cells; type 2: cells with filopodia; type 3: cells with lamellipodia and type 4: fully spread platelets; Figure 5C-D). Surprisingly, the higher number of total adherent cells observed on storage day 4 after RhoA inhibition (Figure 5A) was not correlated with a higher percentage of activated cells (type 2, 3 and 4) compared to cells stored in buffer (Figure 5C). Whereas, the upregulation of PKA induced a significant shift of cell morphology from type 2 to type 1 (resting platelets), in comparison to untreated-CSPs on day 4 (% Platelet phenotype/field mean±SEM: forskolin vs. buffer type 1, 65±18% vs. 31±13%, p=0.0433 and type 2, 21±5% vs. 44±4%, p=0.0176; Figure 5C, respectively). Of note, the inhibition of caspase-9 showed similar spreading phenotypes compared to untreated-CSPs on storage day 4 (Figure 5C). Finally, comparable total number of adherent cells as well as spreading patterns were observed in all treated-CSPs in comparison to cells stored in buffer on storage day 7 (Figure 5B and D).

These data suggest that inhibition of cold-induced GPIb-alpha clustering signaling better conserved the adhesion ability of CSPs, upon 4 days of storage.

Effect of apoptosis inhibition on the kinetic of clot formation

To deeper investigate the hemostatic functions of treated-CSPs, we analyzed the platelet clot formation performing a clot retraction assay. We found that inhibition of either GPIb-alpha clustering or caspase-9 did not impair clot retraction in comparison to cells stored in buffer (Figure 6A and B). In contrast, the extent of platelet clot retraction was almost completely abolished in the presence of the PKA agonist (% Clot retraction mean±SEM: forskolin vs. buffer day 4, 1±0% vs. 88±1%, p<0.0001 and day 7, 1±0% vs. 91±1%, p<0.0001; Figure 6A and B, respectively).
Since clot retraction is a crucial mechanism to stabilize thrombi, we investigated the kinetic of whole blood clot formation performing the thromboelastography assay. We designed an experimental setting that mimics platelet transfusion in thrombocytopenic patients. Briefly, we produced platelets-depleted full blood samples from healthy donors and spiked-in these samples with CSPs. We found that CSPs treated with RhoA or caspase-9 inhibitor maintain similar ability to form clots, in terms of maximum clot firmness (MCF) and maximum lysis (ML) compared to cells stored in buffer at 4°C (Figure 7A, D and 7C, F, respectively). In contrast, a significant decrease of the MCF on day 4 (MCF mean±SEM: forskolin vs. buffer day 4, 45.66±2.93 vs. 58.02±1.12, p=0.0173; Figure 7B) and increase of ML were detected after incubation with the PKA activator indicating reduced stability of the clots (% ML mean±SEM: forskolin vs. buffer day 1, 4±0.3% vs. 23±2%, p=0.0006; day 4, 2±1% vs. 24±4%, p=0.0076; day 7, 2±0.6% vs. 14±4%, p=0.0206; day 10, 2±0.6% vs. 19±4%, p=0.0169; Figure 7E, respectively).

Taken together our data indicate that the inhibition of apoptosis by blocking cold-induced GPIb-alpha- or caspase-9-mediated signals does not affect the contribution of CSP to clot formation or stability.

**Preventing the transduction of the cold-induced GPIb-alpha clustering signal reduces the fast clearance of cold-stored platelets in vivo**

Based on the results of our *in vitro* analyses, which suggest the RhoA inhibitor G04 as the most promising compound, and in accordance with the principles of the 3Rs (Replacement, Reduction and Refinement) for animal research, we investigated whether the survival of CSPs could be improved by preventing cold-induced GPIb-alpha clustering signal transduction. After 7 days of storage, CSPs were administrated into the mice and the survival was analyzed 1, 2, 5 and 24 hours (h) post injection. As shown in Figure 8 significantly higher percentage of treated-CSPs was detected in the mouse circulation 2 and 5 h post injection in comparison to cells stored in buffer (% Survival of human platelets mean±SEM: G04 vs. buffer, 2 h, 47±10% vs. 26±6%, p=0.0387; 5 h, 40±7% vs. 18±3%, p=0.0355; Figure 8). Of note, comparable recovery after injection was observed between platelets stored in buffer and with the RhoA inhibitor (see Supplementary Figure 6).

These data indicate that the inhibition of cold-induced apoptosis by blocking the GPIb-alpha clustering signal improves the survival of CSPs.
Discussion

In the present study, we investigated the impact of apoptosis inhibition on the hemostatic functions and life span of CSPs. Giving the specific target of each inhibitor (Figure 1 and supplementary methods), we found that G04 might be the most promising one, as this agent provided the best compromise between apoptosis inhibition and side effects on platelet functions. We presume that this is due to the fact that G04 blocks the cold-induced apoptosis at an early stage (outside/inside signal transduction). Therefore, it might better maintain the platelet integrity and functionality during cold storage, compared to the other tested inhibitors that target downstream proteins (Figure 1). Interestingly, we found that the inhibition of apoptosis induced by G04, which prevents the transduction of the cold-induced GPIb-alpha clustering signal, improves delta granule release and platelet response to agonists. Furthermore, the ability of platelets to adhere to fibrinogen and to form a stable clot upon exposure to thrombin was conserved. More importantly, we observed a significant improvement of the survival of CSPs upon inhibition of the intracellular signal induced by GPIb-alpha clustering indicating promising potential for clinical use.

The apoptotic phenotype of CSPs was detectable in our study already after 4 days of storage. The blockade of cold-induced apoptosis signaling pathway at three different stages (GPIb-alpha clustering signal, PKA as well as caspase-9, Figure 1) showed protective effect as determined by testing MMP and PS surface externalization. First, we inhibited the transduction of the outside/inside signal induced by GPIb-alpha clustering by blocking the RhoA guanine exchange factor binding domain, which maintains the protein in its inactive form (RhoA-bound GDP).27 In the presence of the inhibitor, we detected better CSPs activation, aggregation and adhesion as well as similar clot retraction and thrombus stability in comparison to untreated cells. Given the key role of RhoA in the cytoskeletal assembly events regulating platelet functionality28, 29, our findings might appear unexpected. This may be explained by the fact that other members of the Rho GTPase family, like Rac1, Cdc42, RhoB and RhoC, have redundant functions in the regulation of platelet functionality.27, 30 Therefore, one or more of these proteins might compensate the inhibition of RhoA. This hypothesis is supported by a previous study, where the RhoA inhibitor does not affect either Rac1 or Cdc42 functions.27 Another possible explanation might be a protective effect regarding the accumulation of PSLs. The inhibition of GPIb-alpha
signaling at a very early stage might decrease PSLs preserving cell integrity and reducing the formation of exhausted platelets.

Next, we used an agent (forskolin) which upregulates PKA via AC activation. One of the downstream targets of PKA is the proapoptotic protein Bad, which is sequestrated upon phosphorylation into the cytoplasm. By this mechanism the translocation of Bad to the mitochondrial membrane is prevented and the apoptosis signaling is inhibited. Despite the well-recognized role of PKA in the intrinsic apoptosis pathway, the impact of the modulation of its activity on platelet functions still remains controversial, likely due to the wide range of downstream targets regulated by PKA-phosphorylation. Several groups observed that enhanced PKA activity prevents apoptosis induction increasing platelet life span \textit{in vivo}.\textsuperscript{24, 32} One study showed, however, that PKA activation correlates with enhanced apoptosis and reduced thrombin-induced platelet activation.\textsuperscript{33} In the present work, CSPs treated with the PKA agonist showed sufficient prevention of apoptosis after cold storage but caused a drastic impairment of cell functions. This is likely due to a higher phosphorylation of one of the PKA downstream targets, vasodilator-stimulated phosphoprotein (VASP), which is known to negatively regulate platelet functionality.\textsuperscript{34, 35} Another important finding of our study is that the clot stability (clot retraction and resistance to fibrinolysis) was impaired in PKA agonist-treated CSPs. This finding might indicate disadvantages for clinical applications of this compound.

In the third part of our \textit{in vitro} study, we blocked the cold-induced apoptotic signal at a late stage by inhibiting the autocatalytic cleavage of caspase-9 (Figure 1). We found comparable platelet responsiveness between caspase-9 inhibitor treated-platelets and cells stored in buffer at 4°C. These data are in line with a previous study that showed normal functionality of caspase-9-depleted murine platelets.\textsuperscript{36}

Taking together our \textit{in vitro} data indicate that apoptosis and activation signaling pathways in CSPs might be better dissected if the signal could be blocked at an early stage. The better responsiveness in the presence of RhoA inhibitor (Supplementary Table 1) suggests additional protective effect against apoptosis-independent PSLs.

RhoA inhibitor has been recently tested in mice.\textsuperscript{37} In this study, RhoA inhibitor was administered daily intraperitoneally at 40 mg/kg for 7 days. All animals survived and no adverse events were observed indicating the safety of this compound. Despite this observation and considering the expected dilution effect after PC transfusion, the removal of G04 from CSPs to reduce potential
systemic toxic effects still remain a relevant question; in particularly in the absence of a clinical study on its safety.

Our in vitro results showed better platelet functions and cell integrity upon G04 incubation, compared to untreated CSPs or those that have been treated with forskolin or caspase-9 inhibitor (Supplementary Table 1). Therefore, we thought to perform an in vivo study and we observed a significantly higher number of circulating CSPs 2 and 5 h after injection, when cells were treated with RhoA inhibitor compared to buffer. This finding shows that the presence of the inhibitor effectively extends the half-life of CSPs. Our in vivo results are in line with a recent study that reported improved survival of CSPs after 14 days of storage upon inhibition of p38MAPK\textsuperscript{38}, which is known to be involved in the apoptotic signal in PCs.\textsuperscript{39}

Nevertheless, our study has some limitations. Although better functionality and survival of RhoA inhibitor-treated CSPs were observed, we did not verify their in vivo functionality. Therefore, further investigations testing the platelet functions in vivo are needed to address this crucial question. Moreover, since the aim of the present study was to screen three apoptosis inhibitors for their efficacy and impact on platelet functions and half-life, we focused on the individual effect. Future studies should, however, investigate the effects of combination of inhibitors during cold storage. Keeping in mind that the final goal would be to add reagent/s to enhance CSP survival without affecting their functionality, the possibility to combine compounds might be a promising approach that should be addressed in future studies. Furthermore, we reported that the higher functionality of refrigerated platelets was even more pronounced upon inhibition of the signal transduction induced by GPIb-alpha clustering. It could be argued that increased risk of thrombosis might exist for patients receiving these products. Even if this consideration is correct and legitime, it can be speculated that in some clinical cases like active bleeding upon injury in thrombocytopenic patients, transfusion of CSPs with better functionality would be more efficient to treat bleeding compared to PCs stored at RT. Although robust data from clinical trials with a significant number of patients are still missing, a recent small pilot study investigated the safety and feasibility of CSPs in patients during cardiothoracic surgery. CSPs have been shown to be still functional after 14 days of storage and no significant difference in clinical outcome was observed compared to standard products. These data indicate toward the feasibility of CSPs application to treat perioperative bleeding.\textsuperscript{40}

In conclusion, our study provides novel insights on the in vitro hemostatic functions and half-life of CSPs upon inhibition of cold-induced apoptotic signaling pathways. Our findings indicate that the combination of cold storage and apoptosis inhibition might provide a promising strategy to
prolong the storage time without affecting cell functionality or reducing platelet survival. Nevertheless, further analysis and clinical studies are still needed to evaluate whether the use of these products might also give better patient outcome.
References


Figure legends

Figure 1: Schematic illustration of the intrinsic apoptotic pathway and the corresponding targets of the apoptosis inhibitors used in the present study.

It is presumed that cold storage of platelets induces clustering of the glycoprotein Ib-alpha (GPIb-alpha) leading to apoptosis which in turn triggers the reduction of the mitochondrial membrane potential (MMP) and phosphatidylserine externalization. In the present study we used the following apoptosis inhibitors: G04 which inhibits RhoA binding its guanine exchange factor domain. Forskolin that enhances the functionality of adenylyl cyclase (AC) inducing conformational change upon its binding and increasing the production of cyclic adenosine monophosphate (cAMP). The latter, triggers the activation of the protein kinase A (PKA) which in turn inhibits the pro-apoptotic protein Bad by phosphorylation. Caspase-9 inhibitor which prevents the autocatalytic cleavage of the pro-caspase 9 protein by a covalent irreversible binding. Abbreviation: Cyto C, cytochrome C.

Figure 2: Apoptosis inhibition during cold storage of platelet concentrates.

The mitochondrial membrane potential (MMP; A-C) and the percentage of apoptotic cells (% Annexin positive cells; D-F) of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols) were detected by flow cytometry after 1, 4, 7 and 10 days of cold storage, respectively. MFI: mean fluorescence intensity. Data are shown as box and whiskers±Standard Error of Mean. *p<0.05; **p<0.01; ns: not significant, n=4.

Figure 3: The impact of apoptosis inhibition on alpha (CD62-P) and delta (CD63) granules release from cold-stored platelets.

The expression of CD62-P (A-C) and CD63 (D-F) on platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols) was analyzed after 1, 4, 7 and 10 days of cold storage, respectively. The expression of both markers was detected by flow cytometry after stimulation with TRAP-6 (10 µM). Data are shown as box and whiskers±Standard Error of Mean. *p<0.05; **p<0.01; ***p<0.001; ns: not significant, n=4.
**Figure 4: Aggregation and agglutination of cold-stored platelets upon apoptosis inhibition.**

The maximal aggregation (A-C) and agglutination (D-F) abilities of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols) were measured after stimulation with the inductors TRAP (20 µM) and ristocetin (1 mg/mL), respectively. Data are shown as box and whiskers±Standard Error of Mean. *p<0.05; **p<0.01; ****p<0.0001; ns: not significant, n=4.

**Figure 5: Cold-stored platelet adhesion to fibrinogen upon apoptosis inhibition.**

The adhesion ability of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols) was measured after TRAP-6 stimulation on storage day 4 (A, C) and 7 (B, D), respectively. The number of adherent cells (A and B) and the percentage of the different platelet phenotypes (type 1: resting cells; type 2: cells with filopodia; type 3: cells with lamellipodia and type 4: fully spreaded cells; C and D) were quantified from 6 different microscopic fields per coverslips, respectively. Representative immunofluorescence images of adherent cells (A and B; scale bar: 20µm) and of the platelet phenotypes (C and D). Green signal, glycoprotein IIb/IIIa (A-D). Data are shown as box and whiskers±Standard Error of Mean. *p<0.05; ns: not significant. C and D, if not indicated the data were not significant. n=4.

**Figure 6: Clot retraction ability of platelet concentrates after cold-induced apoptosis inhibition.**

The percentage of clot retraction of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols), forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols), was analyzed after TRAP-6 stimulation on storage day 4 (A) and 7 (B), respectively. The clot surfaces were calculated as percentage of retraction area compared to the total area. For forskolin a virtual value of 1% was reported in the graphics. Lower panel: representative pictures taken after 1 hour. Data are shown as box and whiskers±Standard Error of Mean. ****p<0.0001; ns: not significant, n=4.

**Figure 7: The impact of cold-induced apoptosis inhibition on the kinetic of clot formation.**

The maximum clot firmness (A-C) and the percentage of maximum lysis (D-F) of platelet concentrates stored at 4°C in buffer (white symbols) or with G04 (RhoA inhibitor; blue symbols),
forskolin (PKA activator; orange symbols) and caspase-9 inhibitor (green symbols) were measured performing thromboelastography assay (extrinsic test), respectively. Data are shown as box and whiskers±Standard Error of Mean. *p<0.05; **p<0.01; ***p<0.001; ns: not significant, n=4.

**Figure 8: Survival of cold-stored platelets upon inhibition of cold-induced GPIb-alpha clustering signal transduction.**

Cold-stored platelets stored for 7 days in buffer (full squares and dashed blue line) or with G04 (RhoA inhibitor; full tringles and continues blue line) were administered into the NSG 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; ns: not significant (n=4).
Figure 6

A  Day 4

% Clot retraction

B  Day 7

% Clot retraction

Buffer  G04  Forskolin  Casp-9 inhibitor

Buffer  G04  Forskolin  Casp-9 inhibitor

ns  **** ns  ns

Images of test tubes with clots from Day 4 and Day 7.
Figure 8

% Survival of human platelets vs. Time after platelet injection (hours)

- CSPs in buffer
- CSPs in G04

* indicates statistically significant difference; ns indicates non-significant difference.
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. 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. 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.

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. 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. 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. 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 °C. Fresh platelets were used as positive control.

**Platelet adhesion**

The adhesion ability of CSPs was assessed as previously described with minor modifications. 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<sup>scid</sup> Il2rg<sup>tm1Wjl</sup>/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. Sex- and age-matched (10-12 weeks) animals were used in this study. Platelets (4x10<sup>9</sup> 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 determined 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.

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.

References