SUPPLEMENTARY APPENDIX

Amotosalen/ultraviolet A pathogen inactivation technology reduces platelet activatability, induces apoptosis and accelerates clearance

Simona Stivala,[1,2] Sara Gobbato,[1,2] Laura Infanti,[3] Martin F. Reiner,[1,2] Nicole Bonetti,[1,2] Sara C. Meyer,[4] Giovanni G. Camici,[5] Thomas F. Lüscher,[6] Andreas Buser[3] and Jürg H. Beer[1,2]

[1]Laboratory for Platelet Research, Center for Molecular Cardiology, University of Zurich; [2]Department of Internal Medicine, Cantonal Hospital Baden; [3]Regional Blood Transfusion Service of the Swiss Red Cross, Basel; [4]Division of Hematology and Department of Biomedicine, University Hospital Basel; [5]Center of Molecular Cardiology, University of Zurich and [6]Department of Cardiology, University Heart Center, University Hospital Zurich, Switzerland

 doi:10.3324/haematol.2017.164137

Received: January 9, 2017.

Accepted: July 13, 2017.

Pre-published: July 20, 2017.

Correspondence: hansjuerg.beer@ksb.ch

Amotosalen/UVA Pathogen Inactivation Technology Reduces Platelet Activatability and Induces Apoptosis and Accelerated Clearance

Simona Stivala, Sara Gobbato, Laura Infanti, Martin F. Reiner, Nicole Bonetti, Sara C. Meyer, Giovanni G. Camici, Thomas F. Lüscher, Andreas Buser and Jürg H. Beer

Supplemental methods

Impedance platelet aggregometry

Platelet samples (approximately 2 ml) from untreated and IBS-treated AU were aseptically taken at day (d) 1, 5 and 7 of storage. Samples were diluted four times with sterile 0.9% NaCl solution to a final volume of 1 ml/test and kept at 37°C with constant stirring on a platelet aggregometer (Chrono-Log, Havertown, PA, USA). Platelet aggregation was induced by addition of equine tendon collagen (5 and 10 μg/ml; Chrono-log, Havertown, PA, USA) or thrombin (0.25 and 0.5 U/ml, Sigma-Aldrich, St. Louis, MO, USA) after 1 min baseline recording and recorded for additional 5 min. Aggregation was expressed as maximal aggregation (in ohm), area under the curve (AUC, ohm x min), lag time (seconds) and slope, and calculated by the AGGRO/LINK® software (Chrono-Log).

Flow cytometry

Platelet samples (1μl) were diluted in 50 μl PBS and incubated with specific anti-human antibodies against GpIbalpha, active integrin 2b3a (PAC-1, both BD Biosciences, San Jose, CA, USA) and P-selectin (eBioscience, San Diego, CA, USA). For the detection of phosphatidylserine/phosphatidylethanolamine, platelets were incubated with APC-conjugated Annexin V (BioLegend, San Diego, CA, USA). After 15 min incubation, reaction was stopped by adding 200 μl PBS and samples acquired on a Fortessa LSRII (BD Biosciences). Desialylation of platelets was determined by incubation of samples with FITC-conjugated *Erythrina Cristagalli* lectin (ECL, EY Laboratories, San Mateo, CA, USA) in PBS for 20 minutes, followed by dilution with 200 μl PBS and immediate analysis.

Glycocalicin ELISA

One ml sample from each AU was spun down at 340 RCF to pellet platelets, and supernatant centrifuged again at 2000 RCF for 20 min followed by a final centrifugation at 17’000 RCF

for 30 min (all steps except first were carried out at 4°C). A flat-bottom plate was coated o.n. with 6D1 antibody (generous gift from Barry Coller, New York, US; 5 μg/ml in 50 mM NaHCO3, pH=8) and extensively washed with PBS-Tween 0.05%. After blocking the plate with 2% BSA/ 5% milk in PBS, 200 μl/well for 1h at room temperature, samples and standards (1:8 and 1:16 dilution in PBS-Tween) were applied to the plate in duplicate and incubated for 1 hour at room temperature. After washing, detection antibody biotinylated Ib-23 was added (from Beat Steiner, Roche, Switzerland) at 1.5 μg/ml final concentration and incubated for 1 hour at r.t. Finally, streptavidin-HRP was added at 1:1000 dilution and incubated for 1 hour, after which the plate was extensively washed before addition of TMB substrate (BD Bioscience, Heidelberg, Germany). Reaction was stopped after 5 minutes by addition of an equal volume of 0.5M sulphuric acid and absorbances read on a Versamax plate reader (Molecular Devices, Sunnyvale, US). Samples concentrations were calculated by the SoftMax Pro 4.8 software (Molecular Devices, Sunnyvale, US) by fitting the standard curve into a 4-parameter curve[1,2].

Western Blotting

To detect Bak, phosphorylated p38 and cleaved caspase 3 by Western blotting, platelet samples supplemented with 1μM PGE1 (Enzo Life Sciences, Farmingdale, NY, USA) were pelleted (340 RCF, 10 min). The supernatant was carefully removed and the pellets were snap-frozen in liquid nitrogen and stored at -80°C until lysis. At the time of analysis, platelet were lysed by addition of 50 μl ice-cold lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl pH 8.0, phosphatase inhibitor cocktail 2 (Sigma-Aldrich) and protease inhibitor cocktail (Complete, Roche, Basel, Switzerland), incubated on ice for 10 min, and membrane debris removed by centrifugation at 15'000 RCF, 10 min. Protein concentration in the supernatant was quantified with the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of protein (50 μg) were separated on a 12% polyacrylamide gel, transferred on a PVDF membrane (Immobilon-FL, EMD Millipore, Billerica, MA, USA) and blocked for 1h with Odyssey blocking buffer (LI-COR, Lincoln, NE, USA). Membranes were probed with the following primary antibodies: anti-phospho p38, anti-p38 (both Cell Signaling Technology, Danvers, MA, USA), anti-GAPDH (EMD Millipore), anti-Bak (Santa Cruz Biotechnology, Dallas, TX, USA), anti-BCL_{XL} (BD Biosciences), anti-cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA), o.n. at 4°C, followed by anti-rabbit or anti-mouse-800 2ry antibody (LI-COR), 1h at room

temperature. Membranes were scanned on an Odyssey Infrared Imaging System (LI-COR) and bands quantified with the Odyssey Application Software.

Immunofluorescence staining

Platelets were washed twice with wash buffer (140 mM NaCl, 26 mM Na_2HPO_4, pH=7.2), resuspended in Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.3 mM Na_2HPO_4, pH=7.4) and allowed to rest at 37°C for 30 min, then 50 μl of the platelet suspension was put on glass coverslips and let to adhere for 30 min. Platelets were washed twice with PBS, then fixed with 4% PFA for 10 min, and incubated with rabbit anti-human Bak (Santa Cruz Biotechnology), mouse anti-human Bcl-$_{XL}$ (BD Bioscience) or mouse anti-human Neu1 (Santa Cruz Biotechnologies) in PBS + 0,1 % Tween 20 o.n. at 4°C. After washing 3x with PBS, coverslips were incubated with anti-rabbit 488 and anti-mouse 647/488 in PBS-Tween, for 1h. Coverslips were mounted on glass slide with fluorescent mounting medium (Dako, Glostrup, Denmark) and imaged under an inverted SP8 confocal microscope (Leica, Wetzlar, Germany) with a 63X (1.4 NA) objective and the Leica Application Suite software. Quantification of fluorescence intensity was performed with the Fiji software[3]. For spleen staining, tissue sections (5 μm) of the central part of the organ were obtained on a Leica CM3050 cryostat, fixed with acetone (30 min), stained with Hoechst 33258 (1μg/ml in PBS, 10 min) and coverslipped. Pictures were taken under an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a 20x/0.70 NA objective and fluorescently-labeled platelet area was calculated with the Bioflux software.

Adhesion to collagen and vWF under flow

Bioflux plates (Fluxion Biosciences, San Francisco, CA, USA) were coated with equine collagen (100 μg/ml, Helena Biosciences, Queensway South, UK) or human vWF (100 μg/ml, Hematologic Technologies, Essex Junction, VT, USA) for 1 hour, then blocked with 0.5% BSA in PBS with Ca^{2+}/Mg^{2+} for 10 minutes. Platelets incubated with calcein (4 μM, Enzo Life Sciences) for 1h were diluted 1:2 with 0.9% NaCl and used for the flow experiments. Images were taken on an inverted microscope with a fluorescein filter (Leica DM IRB) and an objective of 10X/0.22 NA at a rate of an image every 5 sec. Platelet-covered area was calculated with the Bioflux software (Fluxion Biosciences).

RNA Immunoprecipitation (RIP) and qPCR

Circulating platelets were isolated from ACD-anticoagulated blood by centrifugation (200 RCF, 15 min, no brake), the PRP carefully aspirated and centrifuged again to remove contaminating red and white blood cells. Platelets were pelleted after addition of PGE1 (1 μM final concentration) by centrifugation at 340 RCF for 10 min, washed twice with platelet wash buffer (140 mM NaCl, 26 mM Na2HPO4, pH=7.2), then resuspended in 40% autologous plasma/60% Intersol (21.5 mM Na2HPO4, 6.7 mM NaH2PO4, 10 mM Na citrate, 32 mM Na acetate, 77 mM NaCl) and plated in a 24-well plate at 1 ml/well. Samples were pre-incubated with cycloheximide (10 μg/ml), and all samples except non-UV were irradiated with UVA light, followed by room temperature storage on a shaker. Twenty-four hours after UV irradiation, platelets from all samples were lysed and the lysate incubated o.n. at 4 °C with protein A/G agarose beads which were pre-incubated with an antibody against eukaryotic initiation factor 4E (eIF4E, Novus Biological, Minneapolis, US) or a control IgG (Santa Cruz, Dallas, US) as previously described[4]. After extensive washing, RNA was extracted from agarose beads with Quick-RNA Minipreps (Zymo Research, Irvine, US), then used for cDNA synthesis with SYBR Select Master mix (Life Technologies, Carlsbad, US). Quantitative-PCR (qPCR) was performed for Bak and GAPDH with the following primers: Bak forward TGAGTACTTCACCAAGATTGCCACCAG; Bak reverse CCGAAGCCCAGAAGAGCCACCA; GAPDH forward: GGGAAGCTTGTCATCAAT GGA; GAPDH reverse TCTCGCTCCTGGAAGATGGT. For WB, samples were prepared as above, after 24 h platelets pelleted and processed for WB as in the paragraph “Western Blotting”.

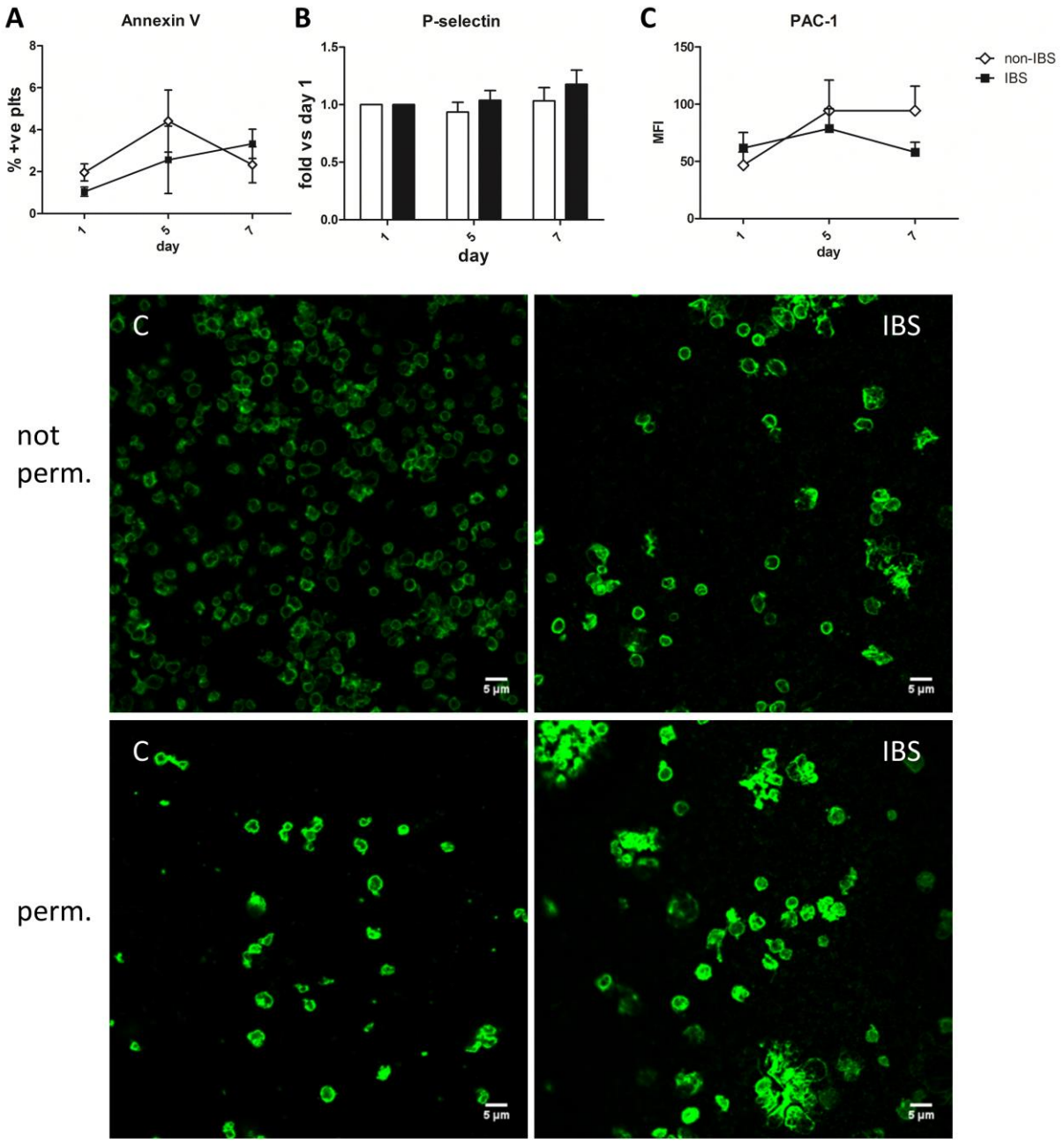
Neuraminidase activity assay

Neuraminidase activity was measured with the Amplite Fluorimetric Neuraminidase assay Kit (AAT Bioquest, Sunnyvale, US) according to the manufacturer’s instructions.

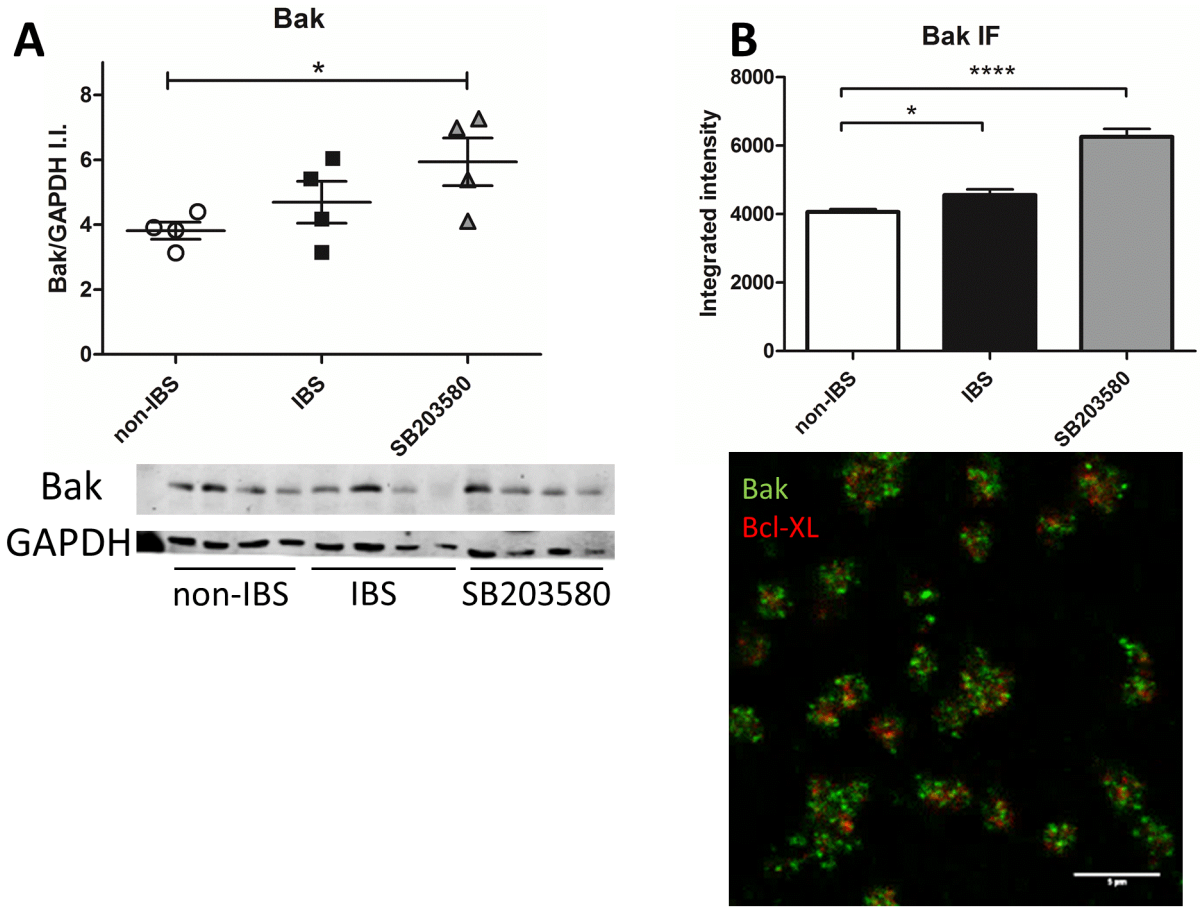
Supplemental figures

	control	IBS	IBS+SB203580	IBS+DANA	p value
plt count	1003.61±238.6	995.57±231	913.83±80.6	873.0±110.1	>0.05
pH	7.43±0.09	7.25±0.14	7.13±0.02	7.11±0.07	<0.0001 C vs IBS <0.005 C vs IBS and C vs SB203580 <0.005 C vs IBS and C vs DANA

Supplementary Table 1 Overall characteristics of platelet concentrates Platelet count and pH at day 1 of storage. Platelet count was not different between groups, while all IBS-treated platelet concentrates show a significant decrease in pH compared to control units, as reported previously by others[5–7].



Supplementary figure 1. The IBS does not induce platelet activation Platelet from non-IBS and IBS samples were stained with APC-conjugated Annexin V (**A**), PE-conjugated P-selectin (**B**) or FITC-conjugated PAC-1 antibody and analysed by flow cytometry. There was no significant difference in Annexin V binding, P-selectin exposure or integrin activation between non-IBS and IBS-treated platelets, suggesting that the procedure did not induce platelet activation. n=15, $p>0.05$. Bottom panel: control or IBS platelets were fixed and stained for Neu1 to detect surface (not permeabilized, upper panels) and total (permeabilized, lower panel) neuraminidase.



Supplementary figure 2. p38 inhibition does not reduce platelet apoptosis induced by the IBS (A) Western Blot analysis of platelets lysate reveals an increased Bak level after the IBS and SB203580 pre-treatment. N=5, $p<0.05$. (**B**) Fixed and permeabilized platelets were stained for Bak (green) and Bcl-$_{XL}$ (red) and the fluorescence intensity determined, confirming an increase in Bak levels. n= 4, *$p<0.05$, **$p<0.01$, ****$p<0.0001$. Scale bar= 5 μm.

References

1. Beer JH, Büchi L, Steiner B. Glycocalicin: A New Assay-The Normal Plasma Levels and Its Potential Usefulness in Selected Diseases. *Blood*. 1994;83(3):691–702.

2. Stivala S, Reiner MF, Lohmann C, et al. Dietary α-linolenic acid increases the platelet count in ApoE-/- mice by reducing clearance. *Blood*. 2013;122(6):1026–33.

3. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat. Methods*. 2012;9(7):676–682.

4. Rufener SC, Mühlemann O. eIF4E-bound mRNPs are substrates for nonsense-mediated mRNA decay in mammalian cells. *Nat. Struct. Mol. Biol.* 2013;20(6):710–7.

5. Jansen G a J, van Vliet HHDM, Vermeij H, et al. Functional characteristics of photochemically treated platelets. *Transfusion*. 2004;44(3):313–9.

6. Picker SM, Oustianskaia L, Schneider V, Gathof BS. Annexin V Release and Transmembrane Mitochondrial Potential during Storage of Apheresis-Derived Platelets Treated for Pathogen Reduction. *Transfus. Med. Hemother.* 2010;37(1):7–12.

7. Van Aelst B, Feys HB, Devloo R, et al. Riboflavin and amotosalen photochemical treatments of platelet concentrates reduce thrombus formation kinetics in vitro. *Vox Sang.* 2015;108(4):328–339.