SUPPLEMENTARY APPENDIX

Hemoglobin interaction with GP1bα induces platelet activation and apoptosis: a novel mechanism associated with intravascular hemolysis

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Supplementary Data:

Materials: Antibodies such as phospho(P)-Lyn (Y507), P-PI3K p85(Y458)/p55(Y199), P-AKT (S473), P-p44/42 MAPK (T202/Y204), Lyn, PI3K, AKT, p44/42 MAPK, caspase-9 and caspase-3 were purchased from Cell Signaling (Beverly, MA, USA); Antibodies for Src (Epitomics, Burlingame, CA, USA), Bak (Abcam, Cambridge, MA, USA) and Bax (Trevigen, Gaithersburg, MD, USA) were purchased. Antibodies for cytochrome C (Sigma Aldrich, St. Louis, USA), β-Actin (Thermo Scientific, Rockford, USA), goat anti-rabbit and goat anti-mouse HRP conjugated antibody (Pierce, Rockford, USA) were purchased. Immunofluorescence antibodies such as anti-human CD62P-FITC (R&D systems, Minneapolis), PE anti-human CD41a and annexin V- FITC antibodies (BD Biosciences), annexin V-PE-Cy5 (Abcam, Cambridge, MA, USA), anti-Hb β (37-8)-PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. The GP1bα monoclonal antibodies were obtained from respective sources such as AN51 (Dako, USA), AK2 (Research Diagnostics, USA), HIP1 (Abcam, Cambridge, MA, USA), SZ2 (Research Diagnostics, USA). The PP2 and PP3 (Calbiochem, Merck Millipore, USA) and U0126 (Promega, Madiason, USA) were purchased.

PNH Patients: Classic PNH patients were recruited through the Hematology Department, All India Institute of Medical Sciences, New Delhi, India. All PNH patients between 14-65 years were recruited as type III PNH patients with exclusion criteria: 1) Blood/plasma/blood cells transfusion within 2 weeks, 2) taking anti-thrombotic/platelet drugs regular basis, 3) chronic infections and 4) any severe life-threatening complications. Plasma was isolated and used for the measuring microparticles and Hb. Freshly isolated platelets were used for measuring platelet surface protein and cellular signaling molecules.

***Glycocalicin purification*:** The Hb binding to immobilized glycocalicin was measured using the SPR described above. Glycocalicin (proteolytic fragment of platelet GP1bα) was purified from outdated human platelets. Briefly, platelets were washed with 13mM sodium citrate, 120mM NaCl, 30mM glucose, pH-7.0, and resuspended in 10mM tris-HCl, 150mM NaCl, 2mM $CaCl_2$, pH-7.4 and subjected to sonication. The suspension was incubated at 37^{o}C for 1 hr and processed for ultracentrifugation at 33,000 rpm for 1 hr at 4^{o}C. The supernatant was loaded on to the lectin wheat germ agarose column (Sigma, USA) and eluted with 2.5% N-acetyl-D-glucosamine in 20mM Tris-HCl, pH-7.4. The eluted fractions were dialyzed with 20mM Tris-HCl, pH-7.4 and processed for ion exchange chromatography using HiTrap DEAE-FF column (GE Healthcare). Glycocalicin was eluted with a linear salt gradient of 0-0.7M NaCl in 20mM Tris-HCl, pH-7.4, and the peak was confirmed by immunoblottingusinganti-GP1b SZ2 antibody. The glycocalicin fraction was dialyzed with 25mM Na_2HPO_4, 100 mM NaCl, pH 8 and concentrated using Amicon centrifugal tubes (MWCO 30 KDa) following which protein concentration was measured using Pierce BCA protein assay kit.

Cytochrome C oxidase assay: The cytochrome C oxidase was measured using kit from Sigma Aldrich (St. Louis, USA). Washed platelets (200 µL) were incubated with different concentrations of Hb (0- 8 µM) at 37^{o}C for 1 hr. The reaction mixture was centrifuged at 13,000 rpm for 3 min. Supernatant was used for measuring cytochrome C oxidase activity. The oxidase activity was determined using spectrophotometer at 550 nm as the ferrocyt c was converted to ferricyt c.

Supplementary Figures and Legends:

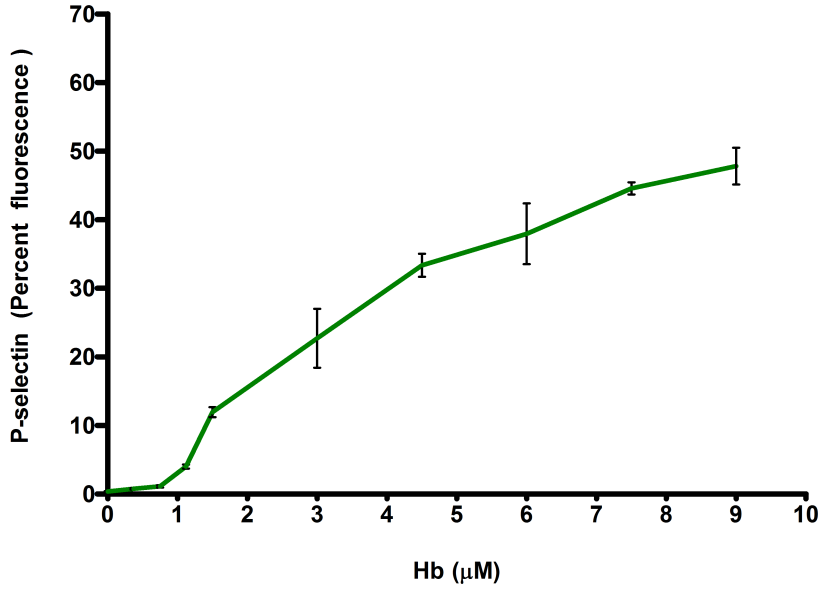


Figure S1. P-selectin expression on platelets in plasma in presence of Hb. Platelets rich plasma was incubated with various concentrations of Hb. Labeled with FITC anti-P selectin antibody and, analyzed by flow cytometry. Data are the mean ± SEM of three experiments. The Hb increased P-selectin expression in a concentration-dependent manner, $P<0.0001$.

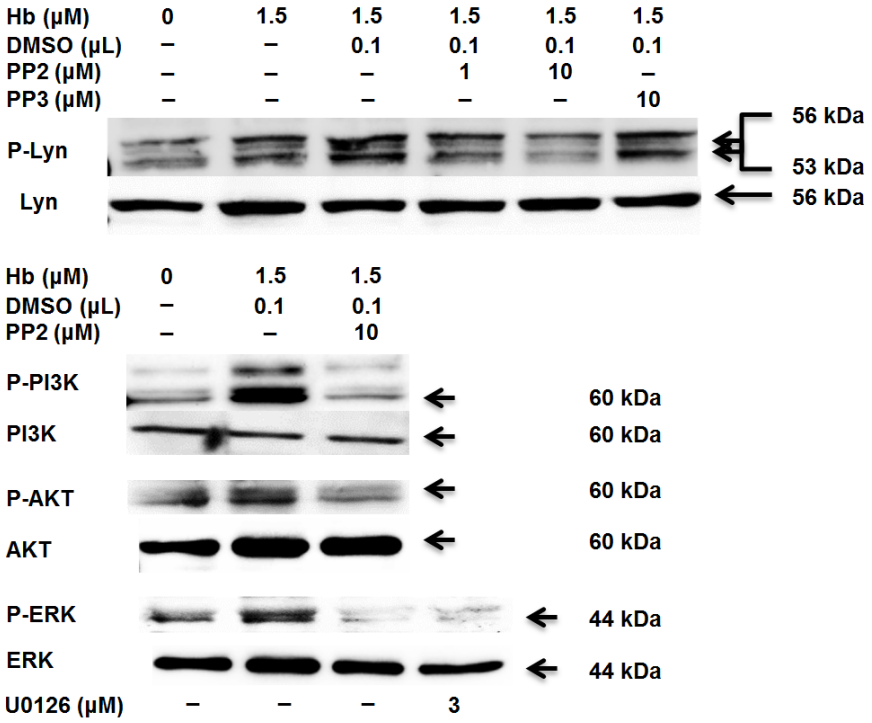
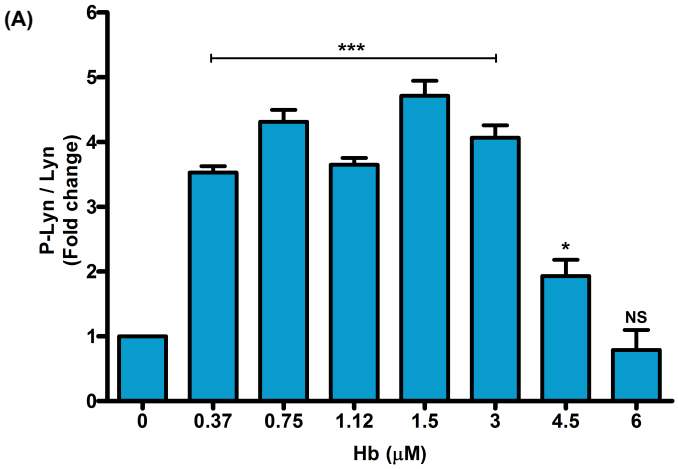
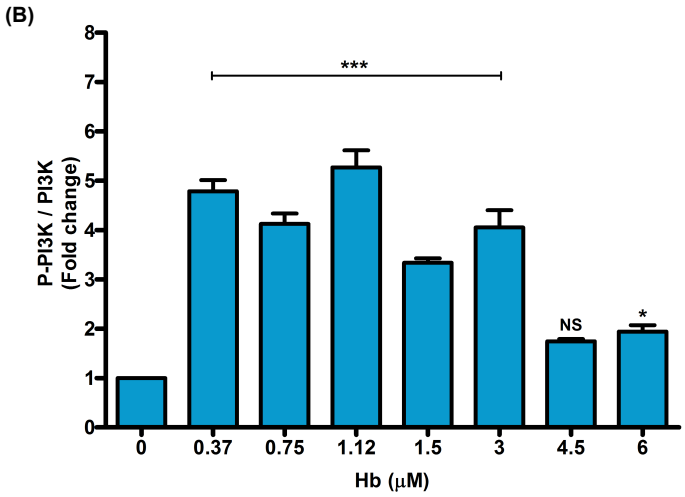
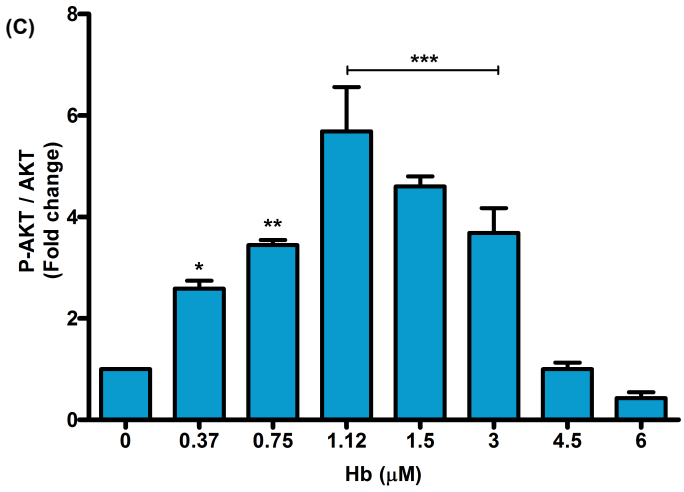


Figure S2. Src inhibitor blocks Hb-mediated platelet activation. Washed platelets were incubated with Hb in presence of Src inhibitor PP2 and control PP3 or ERK inhibitor U0126. The immunoblotting was performed for phospho- and non-phospho Lyn/PI3K/AKT/ERK. The PP2 inhibited the phosphorylation of the above proteins, which was activated by Hb.


(A)
P-Lyn / Lyn
(Fold change)
***
*
NS
0
0.37
0.75
1.12
1.5
3
4.5
6
Hb (μM)


(B)
P-PI3K / PI3K
(Fold change)
***
NS
*
0
0.37
0.75
1.12
1.5
3
4.5
6
Hb (μM)


(C)
P-AKT / AKT
(Fold change)
***
*
**
0
0.37
0.75
1.12
1.5
3
4.5
6
Hb (μM)

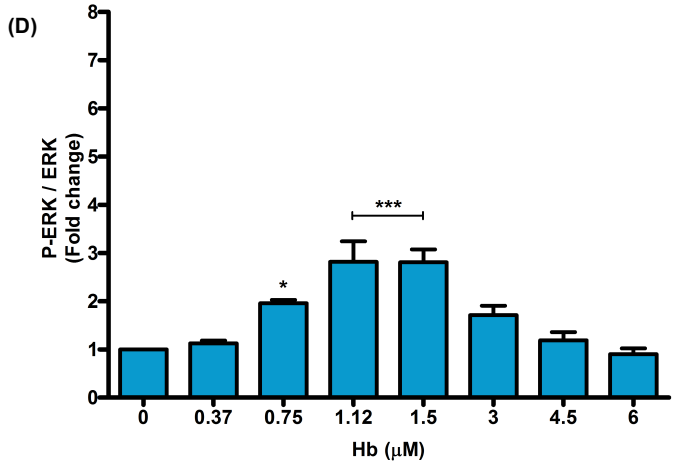
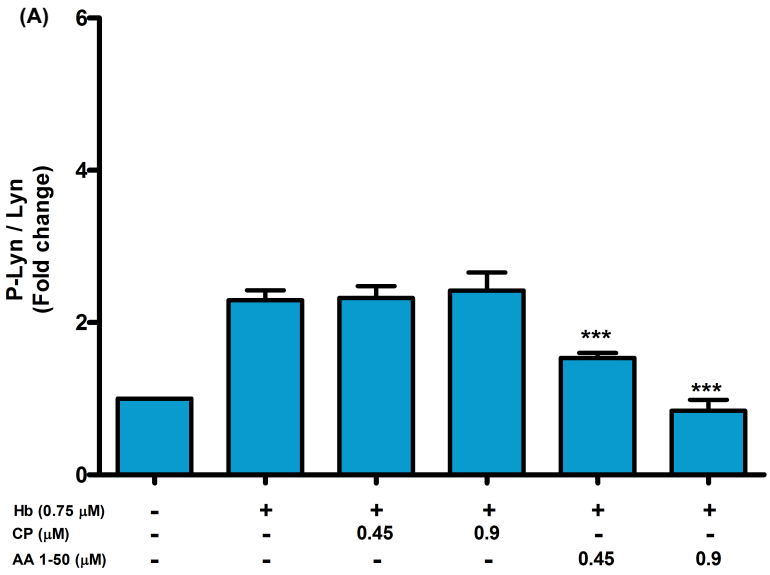
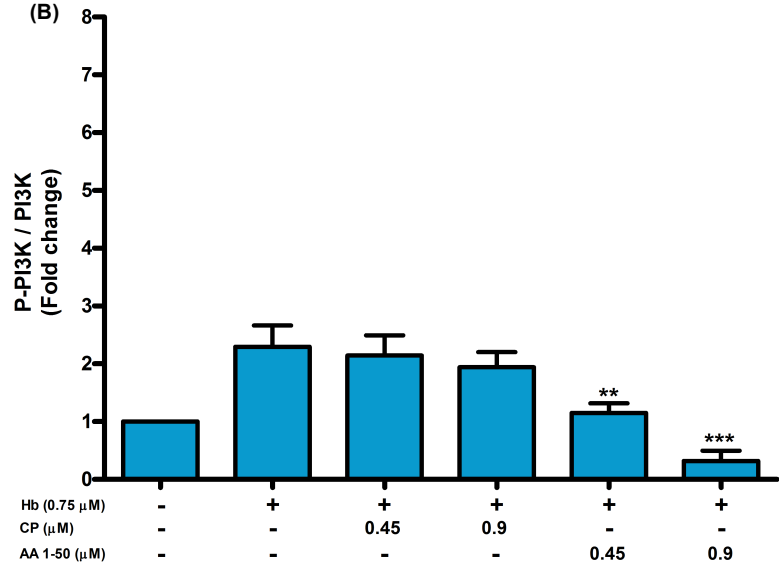
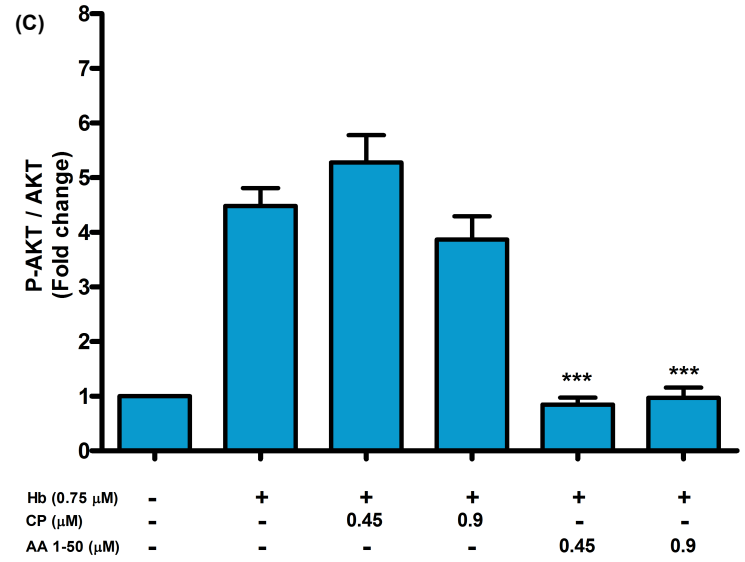


Figure S3. Densitometry ratio of phospho /non-phospho **(A)** Lyn, ***P<0.0001, *P<0.01, NS=non significant (compared to Hb 0μM); **(B)** PI3K, ***P<0.0001, NS, *P<0.01 (compared to Hb 0μM); **(C)** AKT, *P<0.01, **P<0.001, ***P<0.0001 (compared to Hb 0μM); and **(D)** ERK, *P<0.01, ***P<0.0001 (compared to Hb 0μM), as in Figure 2e. Data are the mean ± SEM fold change from three experiments.




(B)
P-PI3K / PI3K
(Fold change)
0
1
2
3
4
5
6
7
8
**
***
Hb (0.75 μM) - + + + + +
CP (μM) - - 0.45 0.9 - -
AA 1-50 (μM) - - - - 0.45 0.9


(C)
P-AKT / AKT
(Fold change)
0
1
2
3
4
5
6
7
8
***
***
Hb (0.75 μM) - + + + + +
CP (μM) - - 0.45 0.9 - -
AA 1-50 (μM) - - - - 0.45 0.9

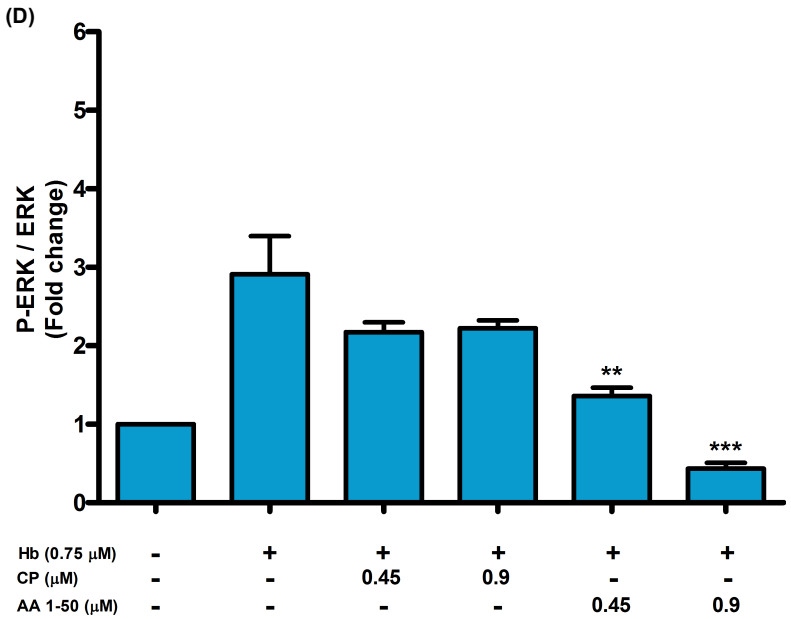

(D)
P-ERK / ERK
(Fold change)
0
1
2
3
4
5
6
**
***
Hb (0.75 μM) - + + + + +
CP (μM) - - 0.45 0.9 - -
AA 1-50 (μM) - - - - 0.45 0.9

Figure S4. Densitometry ratio of phospho /non-phospho **(A)** Lyn, ***P<0.0001, (compared to respective CP conc.); **(B)** PI3K, **P=0.0013, ***P<0.0001 (compared to respective CP conc.); **(C)** AKT, ***P<0.0001, (compared to respective CP conc.); and **(D)** ERK, **P=0.0057, ***P=0.0009 (compared to respective CP conc.), as in Figure 2f. Data are the mean ± SEM fold change from three experiments.

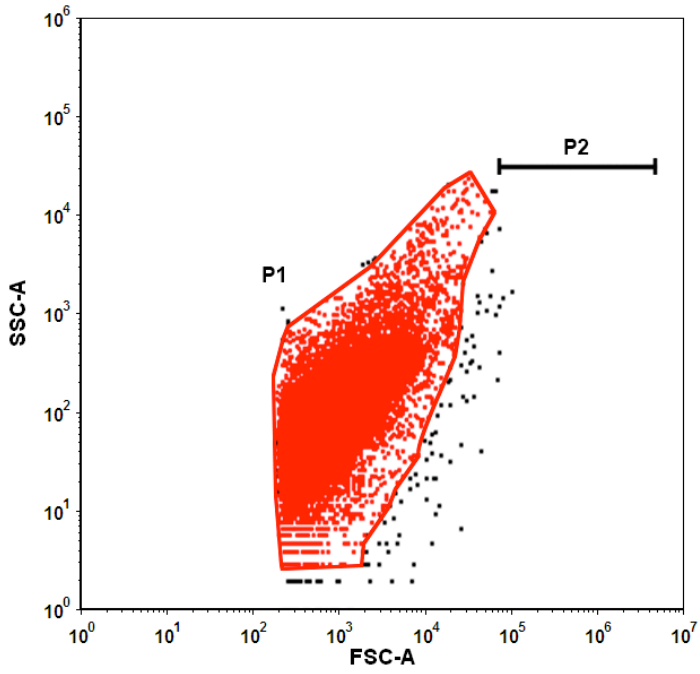


Figure S5. Measuring platelet aggregation. Washed platelets were incubated with various concentrations of Hb and stirred under 1000 rpm in the presence of fibrinogen (500 µg/mL) in total volume of 250µL. In a time interval of 30 sec, 25 µL of platelet aggregates were transferred to 1% paraformaldehyde. Aggregates were measured using flow cytometer. This diagram shows that non-aggregated platelets are gated as P1 and aggregates are in gate P2. Number of events at gate P2 is shown in Figure 3a. The percent aggregated population [aggregated (P2)/ total population (P1+P2) x 100] was calculated and represented as fold change in Figure 3b.

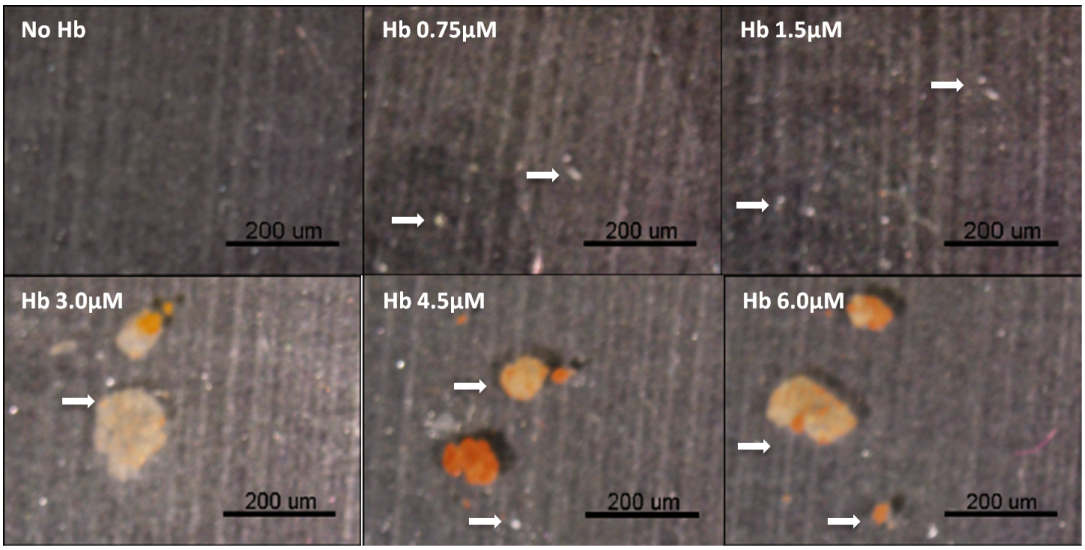


Figure S6. Platelet aggregation in presence of Hb. Washed platelets were incubated with various concentrations of Hb and stirred in presence of fibrinogen (500 µg/mL). The aggregates were measured under microscope, 5X. Hb increased the size of aggregates in a concentration-dependent manner. The arrows indicate the aggregates.

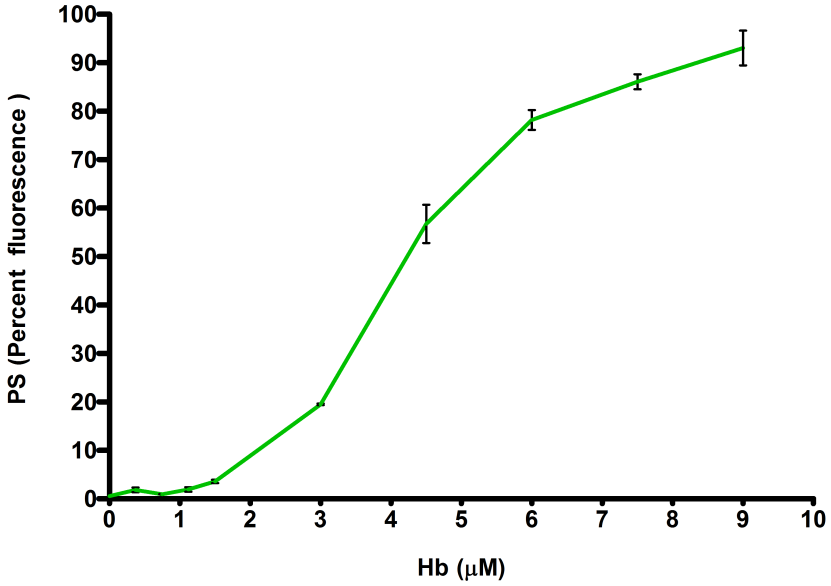
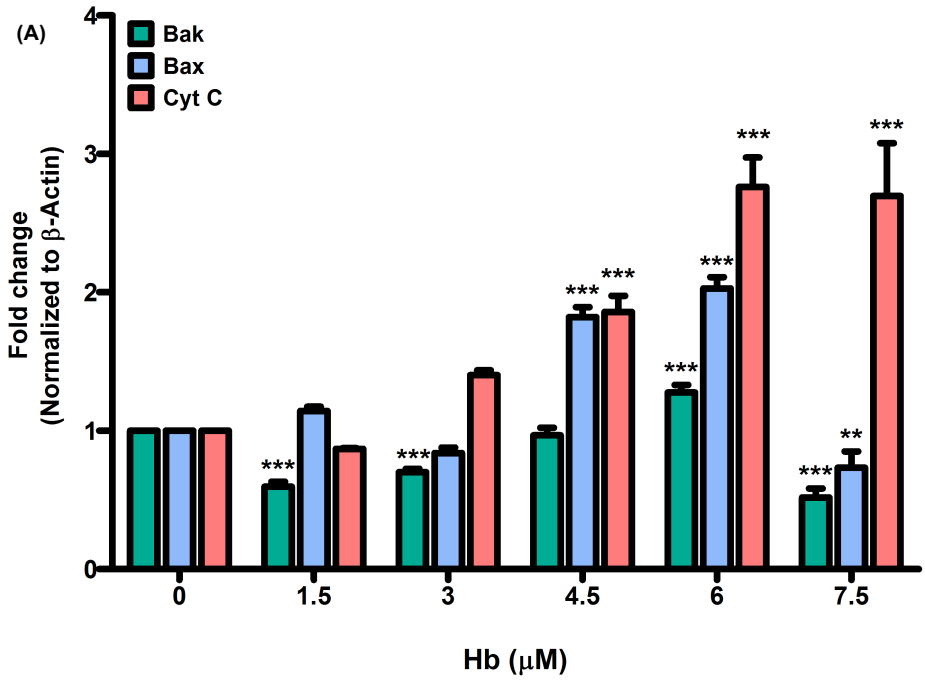


Figure S7. PS expression on platelets in plasma in presence of Hb. Platelets rich plasma was incubated with various concentrations of Hb. Labeled with FITC conjugated annexin V antibody and, analyzed by flow cytometry. Data are the mean ± SEM of three experiments. The Hb increased PS expression in a concentration-dependent manner, $P<0.001$.



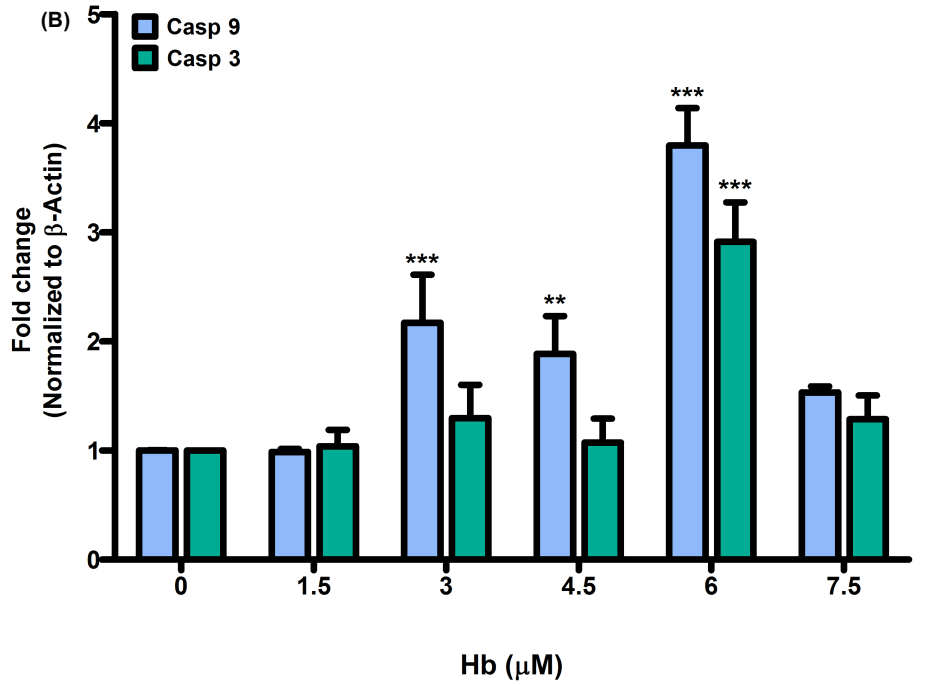
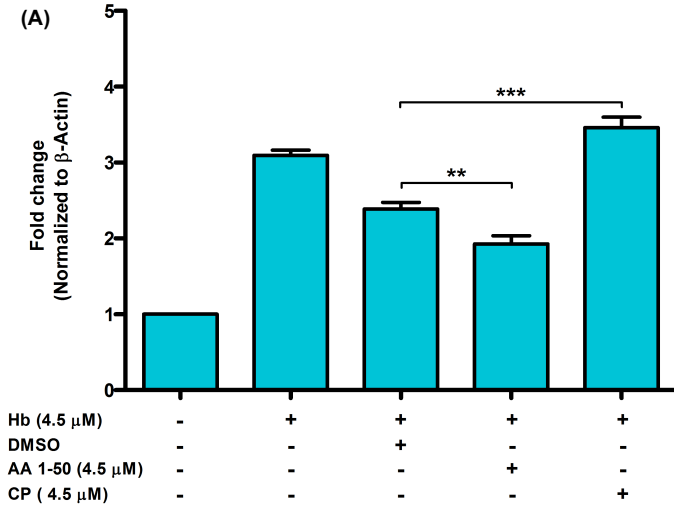
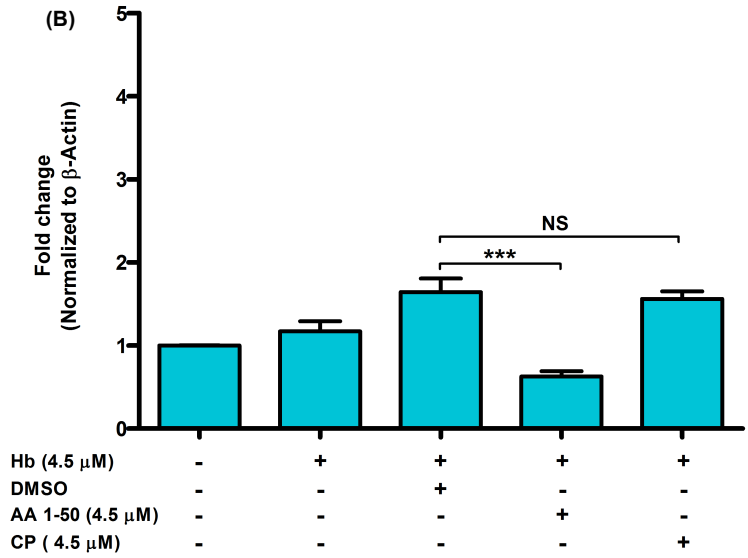


Figure S8. Densitometry ratio of **(A)** Bak, Bax and cytochrome C, **P<0.001, ***P<0.0001, and **(B)** cleaved bands of caspase 9 and caspase 3, **P<0.001, ***P<0.0001, normalized with β-actin from Figure 4b. Data are the mean ± SEM of three experiments.


(A)
Fold change
(Normalized to β-Actin)
0
1
2
3
4
5
***
**
Hb (4.5 μM) - + + + +
DMSO - - + - -
AA 1-50 (4.5 μM) - - - + -
CP (4.5 μM) - - - - +


(B)
Fold change
(Normalized to β-Actin)
0
1
2
3
4
5
NS
***
Hb (4.5 μM) - + + + +
DMSO - - + - -
AA 1-50 (4.5 μM) - - - + -
CP (4.5 μM) - - - - +

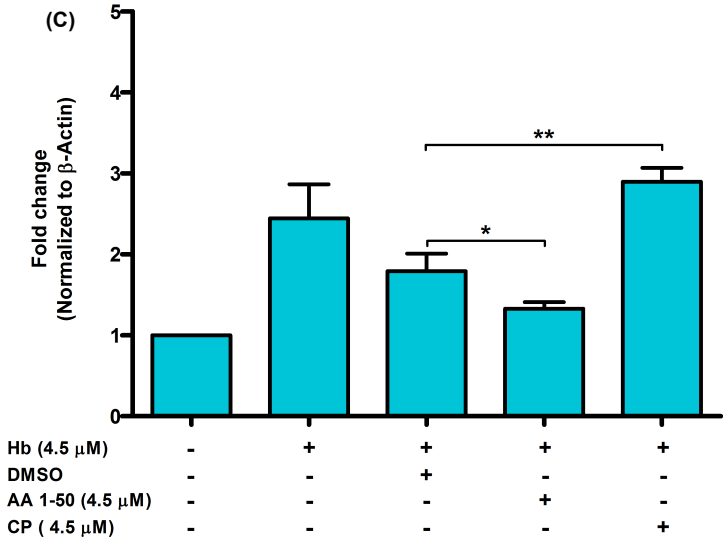

(C)
Fold change
(Normalized to β-Actin)
0
1
2
3
4
5
**
*
Hb (4.5 μM) - + + + +
DMSO - - + - -
AA 1-50 (4.5 μM) - - - + -
CP (4.5 μM) - - - - +

Figure S9. Densitometry ratio of **(A)** Bak, **P=0.0045, ***P=0.0003; **(B)** Bax, ***P=0.0005, NS=non significant; and **(C)** cleaved bands of caspase 9, *P=0.025, **P=0.0023, normalized with β-actin from Figure 4C.Data are the mean ± SEM of three experiments.

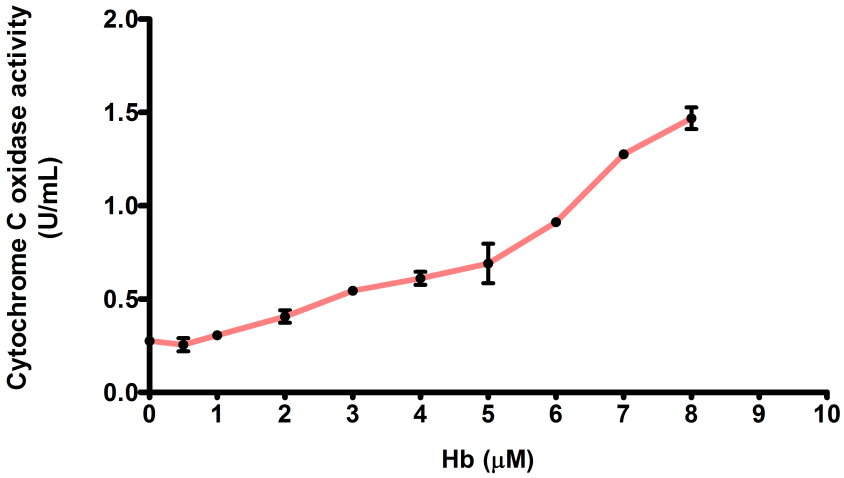


Figure S10. Cytochrome C oxidase activity in platelets in presence of Hb. Washed platelets were incubated with various concentrations of Hb. The Cytochrome C oxidase activity was measured from the supernatant of treated platelets. Data are the mean ± SEM of three experiments. Hb increased the enzyme activity in a concentration-dependent manner, P <0.0001.

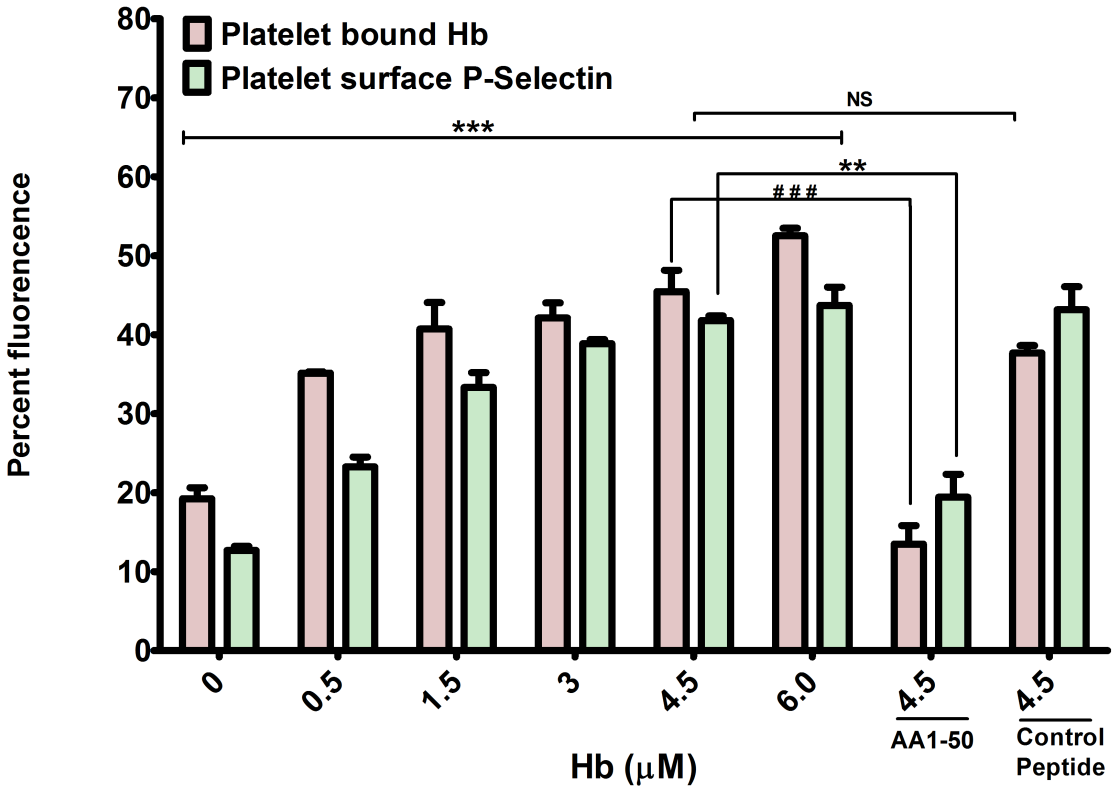


Figure S11. Hb binding to platelets and P-selectin expression in presence of different concentrations of Hb. Washed platelets were incubated for 30 min at 37°C with various concentrations of Hb (0-6 μM). Labeled with FITC anti-P selectin and PE anti Hb antibodies and analyzed by flow cytometry. Data are the mean ± SEM of three experiments. The Hb-binding to platelets and P-selectin expression were increased dose-dependently by Hb, ***$p<0.0001$ one way ANOVA. The equimolar blocking peptide AA1-50 inhibited significantly the effects of Hb (4.5 μM), **$p<0.0017$ and ###$p<0.0009$, t-test.