Supplement Materials and Methods

DMS evaluation

Day 12 megakaryocytes were stained for CD42b and washed with PBS. They were then resuspended in 200 µl PBS and stained for 60 minutes with di-8-ANEPPS (Life Technologies) at a final concentration of 10 µM. Mean fluorescence of PE in the anti-CD42b population was used to quantify the DMS[1].

FV uptake into Megs

Day 12 megakaryocytes were pulse-labeled with FV by incubating with 200 nM of the previously described FV variant35 tagged with Alexa-647 for 1 hour at 37°C as described previously[2]. Excess FV was removed by washing with PBS. Megakaryocytes were analyzed for FV content by flow cytometry.

In vitro proplatelets release potential

Proplatelet extensions were determined using Day 12 megakaryocytes plated on fibronectin-coated dishes (50 µg/ml, Millipore) and incubated overnight in regular culture medium to allow cells to adhere. The next day, consecutively observed attached proplatelet-bearing megakaryocytes were analyzed using phase contrast microscopy. Shafts extending from the main cell body were defined as proplatelet protrusions (PPP), and their number counted per megakaryocyte. Additionally, if the shafts were further branched the branches were defined as proplatelet extensions (PPE) and their number counted per megakaryocyte as well[3,4].

Regular suspension growth conditions were used to isolate EV-PLPs[5]. Day 8 growing megakaryocytes were plated in fresh differentiation medium supplemented with DMSO as vehicle control, or SU6656 or Y27632 or AZD1152, and incubated for 4 days (Figure 1). After that EV-PLP supernatant was obtained by centrifugation at 1000 rpm for 10 minutes. The EV-PLPs were pelleted at 2500 rpm for 10 minutes with 1 µM/ml of prostaglandin E1 (Sigma-Aldrich), and then resuspended in 100 µl of Annexin-V buffer containing anti CD41, CD42a, CD42b and Annexin-V for phenotypic and apoptosis analyses.

Hematocytometer manual megakaryocyte cell counts

At day 8 and 12, the cells were counted using an Improved Neubauer hematocytometer (Fisher Scientific). Nonviable cells were excluded using trypan blue (TB) stain (Sigma-Aldrich, Seelze, Germany). Cells were mixed with TB solution (4:1), and immediately, the number of live (unstained) cells was determined using a light Leica microscope (Leica Microsystems CMS GmbH).

Supplement References

1. Avanzi MP, Goldberg F, Davila J, et al. Rho kinase inhibition drives megakaryocyte polyploidization and proplatelet formation through MYC and NFE2 downregulation. *Br. J. Haematol.* 2014;164(6):867–876.
2. Sim X, Jarocha D, Hayes V, et al. Identifying and enriching the platelet-producing human stem cell-derived megakaryocytes using factor V uptake. *Blood*. 2017;
3. Kawaguchi T, Hatano R, Yamaguchi K, et al. Fibronectin promotes proplatelet formation in the human megakaryocytic cell line UT-7 / TPO. *Cell Biol. Int.* 2012;36:39–45.
4. Foudi A, Kramer DJ, Qin J, et al. Distinct , strict requirements for Gfi-1b in adult bone marrow red cell and platelet generation. *J. EXP. Med.* 2014;211(5):909–927.
5. Takayama N, Nishikii H, Usui J, et al. Generation of functional platelets from human embryonic stem cells in vitro via ES-sacs, VEGF-promoted structures that concentrate hematopoietic progenitors. *Blood*. 2008;111(11):5298–5306.

Supplement Table and Figures legend

Supplement Table 1. List of antibodies used in this paper.
List of antibodies and their uses in this study.

Supplement Figure 1. Maturation of the in vitro-grown megakaryocytes in the presence of inhibitors as measured by DMS and FV uptake.
(**A**) through (**C**) represent effects of drugs on the development of the DMS in in vitro-grown megakaryocytes as measured by MFI and expressed as % of control. (A) demonstrates MFI of DMS in all $CD42b^+$ megakaryocytes, (B) and (C) demonstrate the same evaluation per $CD42b^+$ megakaryocyte size (FSC) or granularity (SSC), respectively. All results are expressed as % of the control (100%). Mean ± 1 SEM are shown with N = 7 independent studies per arm. * = $p \leq 0.05$ compared to control, by one-way ANOVA analysis. (**D**) through (**F**) represent effects of drugs on uptake of FV into in vitro-grown megakaryocytes as measured by MFI and expressed as % of control. (D) demonstrates MFI of FV uptake in all $CD42b^+$ megakaryocytes, (E) and (F) demonstrate the same evaluation per $CD42b^+$ megakaryocyte size (FSC) or granularity (SSC), respectively. All results are expressed as % of the control (100%). Mean ± 1 SEM are shown with N = 5 independent studies per arm. * = $p \leq 0.05$ compared to control, by one-way ANOVA analysis.

Supplement Figure 2. Influence of inhibitors on the quality of in vivo-released platelets from megakaryocytes infused into NSG mice.
(**A**) Percent of TO^+ circulating platelets representing the released platelets from infused either control (open circles) or drug-treated (colored squares) megakaryocytes. (**B**) Percent of Annexin-V^+ circulating platelets representing the released platelets from infused either control or drug-treated megakaryocytes. In (A) and (B), mean ± 1 SEM are shown with N ≥ 6 independent studies per arm. * = $p \leq 0.05$ compared to control, by one-way ANOVA analysis.

Supplement Figure 3. Dose-dependent response of in vivo-released platelets from infused megakaryocytes after thrombin stimulation.
Whole murine blood isolated four hours after hdPlts or human megakaryocytes were infused into NSG mice. (**A**) and (**B**) are mean ± 1 SEM of (A) PAC-1 binding MFI and (B) CD62P surface expression MFI pre- and post a range of low to high dose thrombin on the human platelets in whole murine blood 4 hours after the initial infusion. Gray circles = infused hdPlts. Open circles = infused control megakaryocytes. Colored squares = infused drug-treated megakaryocytes. N =3 independent studies for thrombin range

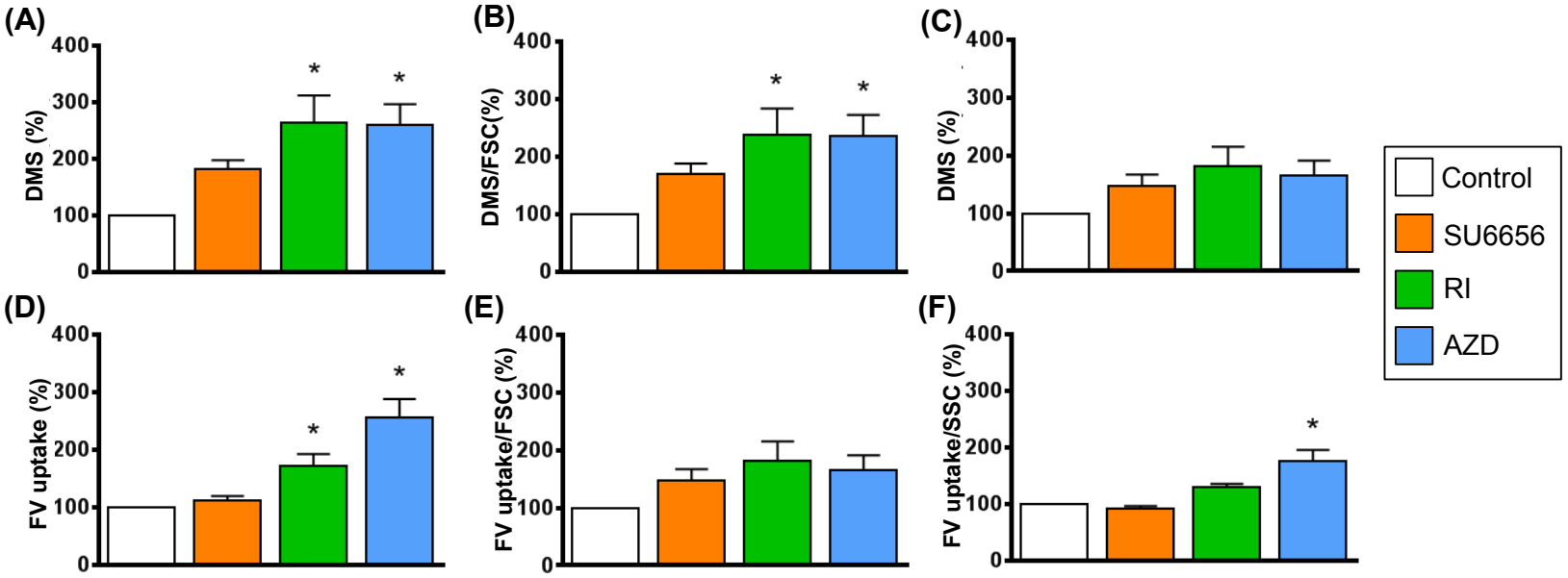
dose for each arm. * = $p<0.05$ compared to hdPlts.

Suppl Tab 1

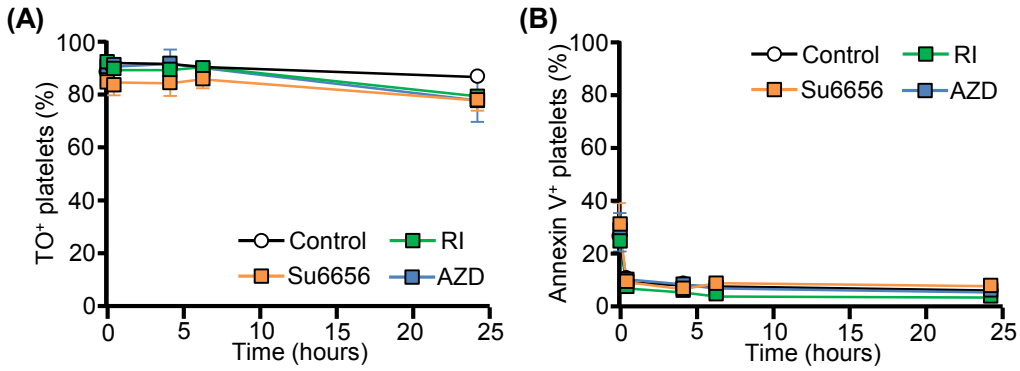
Target	Source	Reactivity	Label	Company	Catalog number	Usage
	Monoclonal mouse	Human	APC	BD Biosciences	559777	Flow cytometry
CD42a	Monoclonal mouse	Human	BV421	BD Biosciences	565444	Flow cytometry
CD42b	Monoclonal mouse	Human	PE	BD Biosciences	555473	Flow cytometry
Annexin V	Bacteria	Human	FITC	BD Biosciences	556420	Flow cytometry
Annexin V	Bacteria	Human	V450	BD Biosciences	560506	Flow cytometry
PAC-1	Monoclonal mouse	Human	FITC	BD Biosciences	340507	Flow cytometry
CD62P	Monoclonal mouse	Human	BV421	BD Biosciences	564038	Flow cytometry
CD41 MWReg30	Rat Fab2	Mouse	AF 647	BD Biosciences	553847	Intra-vital injury
DNA	DNA dye	Nucleic acids	Violet stain	Invitrogen	V35003	Flow cytometry
DNA, RNA	(Tiazole Orange) dye	Nucleic acids	green	BD Biosciences	349204	Flow cytometry

AF Alexa Fluor
PE R-phycoerythrin
APC Allophycocyanin
FITC fluorescein isothiocyanate

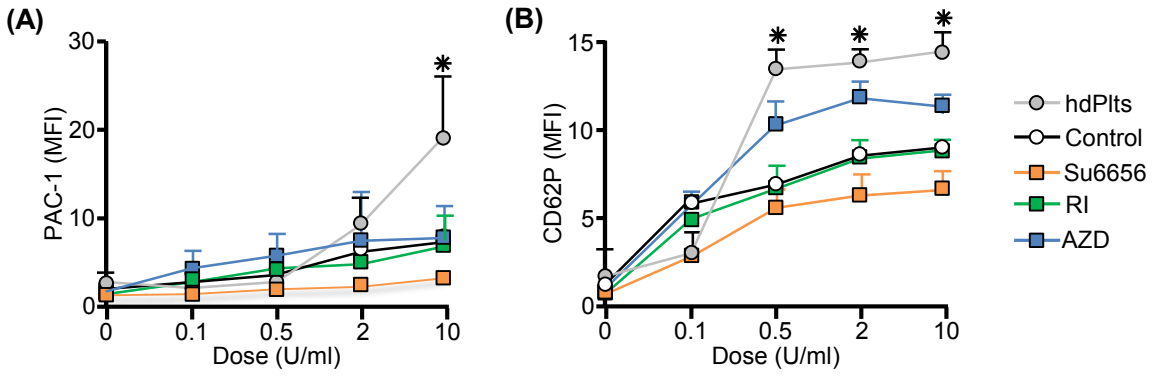
Suppl Fig 1


(A)
DMS (%)
400
300
200
100
0
*
*
(B)
DMS/FSC(%)
400
300
200
100
0
*
*
(C)
DMS (%)
400
300
200
100
0
(D)
FV uptake (%)
400
300
200
100
0
*
*
(E)
FV uptake/FSC (%)
400
300
200
100
0
(F)
FV uptake/SSC (%)
400
300
200
100
0
*
Control
SU6656
RI
AZD

Suppl Fig 2


(A)
TO+ platelets (%)
100
80
60
40
20
0
0
5
10
15
20
25
Time (hours)
Control
RI
Su6656
AZD
(B)
Annexin V+ platelets (%)
100
80
60
40
20
0
0
5
10
15
20
25
Time (hours)
Control
RI
Su6656
AZD

Suppl Fig 3


(A)
PAC-1 (MFI)
30
20
10
0
0
0.1
0.5
2
10
Dose (U/ml)
*
(B)
CD62P (MFI)
15
10
5
0
0
0.1
0.5
2
10
Dose (U/ml)
*
*
*
hdPlts
Control
Su6656
RI
AZD