Supplemental Material

Manuscript title
Effective treatment of edema and endothelial barrier dysfunction with imatinib

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Supplemental Methods

Reagents
Imatinib (as used for *in vitro* experiments) was purchased from ChemieTek (Indianapolis, IN) and dissolved in dimethylsulphoxide (DMSO) to a stock concentration of 10mM. Imatinib mesylate (used for *in vivo* experiments) was purchased from SelleckChem (Houston, TX) and dissolved in phosphate-buffered saline (PBS) to a stock concentration of 5mg/mL. Tyrphostin AG1296 was obtained from Sigma Aldrich (Steinheim, Germany), Y27632 from Tocris Cookson Ltd (London, United Kingdom), BAPTA-AM from Calbiochem (EMD/Merck, Darmstadt, Germany). Thrombin was purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). VEGF was from Invitrogen (Camarillo, CA), histamine from Tocris Bioscience (Ellisville, MO). Thrombin Receptor Activating Peptide (TRAP, TFLLRN) was from dr. S.M. Vogel. Small interference RNAs (siRNAs) against c-Abl, PDGFR-α and -β, Arg, c-KIT, DDR-1 and scrambled RNA (scRNA) were from Santa Cruz Biotechnology (Santa Cruz, CA). The following antibodies were used: anti-c-Abl (#2862), anti-PDGFR-α (#3174) and -β (#3169), anti-pTyr207 CrkL (#3181), anti-CrkL (#3182) and anti-DDR-1 (#5583), all from Cell Signaling Technologies (Danvers, MA), anti-β-actin (Sigma Aldrich), anti-VE-cadherin (SC-6458, Santa Cruz), anti-β-catenin (clone 8E7, Upstate/Millipore, Temecula, CA), anti-pTyr118 Paxillin (44722G, Invitrogen) and anti-Arg (NBP1-18875, Novus Biologicals, Littleton, CO).

Endothelial cell culture
*Human pulmonary microvascular cells* were isolated from human lung tissue, as previously described. Cells were cultured in EGM2-MV culture medium (EBM2 medium supplemented with 5% foetal bovine serum, human epidermal growth factor, fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, hydrocortisone, ascorbic acid, gentamicin and amphotericin according to the manufacturers protocol [BioWhittaker/Lonza, Verviers, Belgium], and with penicillin [100U/mL] and streptomycin [100µg/mL]) and seeded on gelatine-coated 25cm² culture flasks. Cells were grown to confluence at 37°C and 5% CO₂, with a change of culture medium every other day. They were extensively characterized as endothelial cells by the presence of endothelial markers and the absence of epithelial, lymphatic and smooth muscle cell markers. Cells were cultured up to passage 7, for experiments passage 4-7 cells were used.
Human skin microvascular endothelial cells were isolated from human foreskin, as previously described, and cultured with EGM2-MV culture medium. Cells were grown to confluence at 37°C and 5% CO₂, with a change of culture medium every other day, and extensively characterized as endothelial cells.

For human umbilical vein endothelial cells, umbilical cords from healthy donors were obtained from the Amstelland Ziekenhuis, Amstelveen. Cells were isolated, and extensively characterized as previously described. After isolation, cells were resuspended in M199 medium (Biowhittaker/Lonza), supplemented with penicillin 100U/mL and streptomycin 100µg/mL (Biowhittaker/Lonza), heat-inactivated human serum 10% (Sanquin Blood Supply, Amsterdam, The Netherlands), heat-inactivated new-born calf serum 10% (Gibco, Grand Island, NY), crude endothelial cell growth factor 150µg/mL (prepared from bovine brains), L-glutamine 2mmol/L (Biowhittaker/Lonza), and heparin 5U/mL (Leo Pharmaceutical Products, Weesp, The Netherlands). Cells were cultured at 37°C and 5% CO₂, with a change of culture medium every other day. Cells were cultured up to passage 2, for experiments passage 1-2 cells were used. Unless indicated otherwise, experiments were performed with human umbilical vein endothelial cells.

The use of human tissue for isolation of endothelial cells was approved by the Medical Ethical Committee of the VU University Medical Center. Patients gave informed consent for the use of tissue for research purposes.

Transfections
Endothelial cells were transfected with Amaxa Technology (Amaxa Biosystems, Lonza), according to the manufacturers protocol. 80-90% confluent cells were trypsinized and transfected with indicated siRNAs. Transfected cells were seeded on gelatine-coated ECIS arrays or 5cm² culture wells. For control of transfection efficiency, confluent transfected cells were lysed 48h after transfection. 20µg of total protein/condition was electrophoresed, transferred to nitrocellulose membranes and immunoblotted for c-Abl, Arg, PDGFR-α and -β, c-KIT and DDR-1 to analyze protein expression (Supplemental Figure S4I,J).
**Plasmids & Viral transduction**

Lentiviral particles were isolated from the supernatant of HEK293 cells transiently transfected with third-generation packaging constructs and lentiviral expression vectors encoding Paxillin-mCherry (gift of Dr. Olivier Pertz, University of Basel, Switzerland) or VE-cadherin-GFP. HUVECs were infected with supernatant containing lentiviral particles in the presence of 8 µg/ml polybrene overnight.

**Immunofluorescence imaging**

Cells were seeded on glass coverslips coated with glutaraldehyde 0.5% (Fluka, St. Gallen, Switzerland) -crosslinked gelatine. Cells were seeded in 1:1 density and grown to confluence in 48-72 hours. For pretreatment, culture medium was changed to 1% human serum albumin (HSA, Sanquin Blood Supply) in M199, containing pharmacological inhibitors or vector. After 60min of pretreatment, thrombin was added to the wells for a final concentration of 1U/mL. At indicated time points, medium was replaced by 2% paraformaldehyde (37°C, Sigma Aldrich), followed by 15min incubation on ice. Cells were permeabilized with Triton X-100 0.05% (Sigma Aldrich) in PBS, and incubated overnight with primary antibodies against β-catenin, VE-cadherin or pY118-Paxillin. Subsequently, cells were washed and incubated with FITC- or Cy3-labeled secondary antibodies (Invitrogen, Paisly, United Kingdom) and rhodamine/phalloidin (Invitrogen) for 1h at room temperature. Cells were washed, and sealed with Vectashield mounting medium containing DAPI (Vector Laboratories Inc, Burlingham, CA) for nuclear staining. Imaging was performed with an Axiovert 200 Marianas™ inverted wide-field fluorescence microscope, using a 63x Zeiss oil objective (NA 1.4). Images were analyzed with Slidebook software (Intelligent Imaging Innovation, Denver, CO) and ImageJ (National Institutes of Health). For quantification of focal adhesions, pY118-Paxillin images were equally adjusted for contrast. After conversion to black/white images, the number of focal adhesions was counted with the Analyze Particle module in ImageJ.

**Live-Cell Imaging**

For live-cell microscopy, cells were plated on Lab-Tek chambered 1.0 borosilicated coverglass slides coated with 3 mg/ml fibronectin. Cells were imaged using a widefield microscope (NIKON Eclipse TI) with a 60x Apo TIRF oil objective (NA 1.49) and an EMCCD camera (Andor). Images were enhanced for display with an unsharp mask filter and by adjusting brightness and contrast settings in ImageJ.
**RhoA and Rac1 activity assay**

For analysis of RhoA and Rac1 activity, 5cm\(^2\) confluent cells/condition were pretreated with imatinib (10µM) or vector (DMSO 0.1%) and stimulated with thrombin (1U/mL) during indicated intervals. After stimulation, cells were washed with ice-cold PBS and lysed with lysis buffer provided with the G-LISA Activity Assay kit (Cytoskeleton Inc, Denver, CO). After centrifugation of the cell lysates, the supernatant was snap-frozen and stored at -80°C. RhoA and Rac1 activity was determined with the G-LISA RhoA and Rac1 Activity Assay, according to the manufacturers protocol.

**Telemetry measurement of systemic arterial blood pressure**

To assess whether imatinib affects transendothelial passage through effects on systemic blood pressure, male Balb/c mice (strain ByJ, Charles River, 25-30gr) were anesthetized with isoflurane (2-2.5% v/v). After induction of anesthesia, a telemetric device (Type: TA11PA-C10, Data Science International, St. Paul, MN) was inserted in a subcutaneous pocket with a pressure-sensing probe positioned in the aortic arch (via the internal carotid artery). After two weeks of recovery blood pressure measurements were initiated, followed by induction of anesthesia according to the protocol of the Miles assay. Upon induction of anesthesia with fentanyl, midazolam and acepromazine, the blood pressure was measured for 45 minutes to allow normalization of the blood pressure changes induced by anesthesia. After 45 minutes, imatinib mesylate was administered intraperitoneally (20mg/kg, 5mg/mL in PBS), and blood pressure was monitored for 60 minutes to evaluate effects of imatinib on systemic arterial blood pressure.

**Measurement of microvascular perfusion**

To assess whether imatinib affects transendothelial passage through effects on microvascular perfusion, we quantified skin perfusion in C57/BL6J mice (20-30 gr) by contrast-enhanced ultrasonography. Mice were anesthetized with fentanyl, midazolam and acepromazine, followed by cannulation of the right jugular vein (t = 0min) and shaving of the right hind limb. An echo probe was positioned perpendicular to the right hind limb, for cross sectional ultrasound analysis of the proximal adductor muscle group and the overlying skin. After surgery a baseline ultrasound recording (t = 45min) was performed (Sequoia 512, Siemens Medical Systems, Germany); ultrasound recording was maintained throughout the rest of the experiment. The baseline ultrasound recordings were followed by continuous infusion of phosphatidylcholine/polyethylene glycol stearate-coated decafluorobutane-
filled microbubbles, which were manufactured at the Erasmus University Rotterdam as described. Microbubbles were infused via the right jugular vein at an infusion rate of 5 µl/min. Two to three minutes after initiation of microbubble infusion a steady state was reached (t = 60min). To quantify perfusion of skin and muscle in detail, contrast replenishment curves were generated by low-frequency (5 MHz, 1sec) microbubble destruction followed by continuous high-frequency (14 MHz) imaging of microbubble replenishment. Replenishment curves were fitted using the one phase exponential association equation Y=A*(1-exp(-β*t)), in which Y is the microbubble signal and t is time. The value A is the maximal Y value, representing blood volume, whereas the β is a rate constant, representing the red blood cell velocity. Contrast replenishment curves were generated in triplo. To specifically quantify perfusion of the microcirculation, the contrast signal from large arteries was eliminated. This was achieved by subtraction of the contrast signal recovered during the first 0.5 seconds after microbubble destruction. After measuring perfusion under control conditions, imatinib mesylate (50mg/kg in PBS) was administered intraperitoneally (t = 90min) in each mouse. Thirty minutes after imatinib administration (t = 120min) ultrasound measurements described above were repeated. The perfusion parameters were calculated for both skin and muscle, by quantifying contrast signal in different regions of interest during image analysis. Ultrasound data were analyzed off-line using MatLab and GraphPad Software; the ultrasound signal was corrected for differences in blood microbubble density by dividing the skin or muscle microbubble signal by the ultrasound signal in the femoral artery. Triplo measurements were averaged for each mouse.

References

Supplemental Figures

Supplemental Figure S1

Supplemental Figure S1 Dose-dependent inhibition of thrombin-induced macromolecule passage by imatinib. Horse radish peroxidase (HRP) passage over human umbilical vein endothelial cell monolayers under basal and stimulated (thrombin 1U/mL) conditions. Monolayers were pretreated with increasing concentrations of imatinib. Average values of n = 4 experiments, which all showed the same effect.
**Supplemental Figure S2**

Immunofluorescence staining of the adherens junction protein VE-cadherin (green) and nuclei (blue) in human umbilical vein endothelial cells after 30 minutes of thrombin (1U/mL) stimulation. Arrows indicate loss of junctions and presence of intercellular gaps. Scale bars represent 10µm. Representative images of n = 3 experiments.
**Supplemental Figure S3** Barrier protective effects of imatinib in microvascular endothelial cells isolated from human foreskin. Absolute endothelial electrical resistance of confluent human foreskin microvascular endothelial cell monolayers during thrombin 1U/mL stimulation (n = 3 donors).
Supplemental Figure S4 (continued)

**Supplemental Figure S4** Knock-down of the imatinib sensitive kinases in human umbilical vein endothelial cells (HUVECs). (A) Absolute endothelial electrical resistance of HUVECs transfected with scRNA or siRNA against PDGFR-α, PDGFR-β or PDGFR-α/-β. The endothelial resistance was measured under basal conditions and during thrombin (1U/mL) stimulation. Average values of n = 4 experiments. (B) The thrombin response (% decrease in resistance) in wild type versus PDGFR-α, PDGFR-β or PDGFR-α/-β depleted endothelial cells. No statistical difference was observed (n = 4). (C) Absolute endothelial resistance of HUVECs transfected with scRNA or siRNA against c-Abl during thrombin stimulation (n = 4). (D) The thrombin response (% decrease in resistance) in wild type versus c-Abl-depleted endothelial cells. No statistical difference was observed (n = 4). (E) Absolute endothelial resistance of HUVECs transfected with scrambled RNA or siRNA against c-KIT during thrombin stimulation (n = 3). (F) The thrombin response (% decrease in resistance) in wild type versus c-KIT-depleted endothelial cells. No statistical difference was observed (n = 3). (G) Absolute endothelial resistance of HUVECs transfected with scrambled RNA or siRNA against DDR-1 during thrombin stimulation (n = 4). (H) The thrombin response (% decrease in resistance) in wild type versus DDR-1-depleted endothelial cells. No statistical difference was observed (n = 4). (I) Expression of PDGFR-α and -β. HUVECs were transfected with scrambled RNA or siRNA against PDGFR-α and -β and grown to confluence (24h). As no PDGFR protein could be detected, confluent cells were serum starved for 24h with medium containing 1% (1%s) or 2% (2%s) new-born calf serum, or cultured on normal growth medium (cM199). After 24 hours cells were lysed and cell lysates were analyzed for expression of PDGFR-α or -β by Western Blot. Lysates of human lung fibroblasts (FB) served as
positive control for PDGFR expression. M = marker. Representative blots of n = 3 experiments. (J) Expression of Abl-related gene (Arg), c-Kit, c-Abl and DDR-1 in HUVECs treated with scrambled RNA versus siRNA against Arg, c-Kit, c-Abl and DDR-1, respectively. Cells were transfected with scrambled RNA or the indicated siRNA and grown to confluence (48h). Lysates of confluent cells were analyzed for protein expression by Western Blot. c-Abl served as loading control during Arg knock-down to exclude aspecificity of the siRNA.
Supplemental Figure S5 Computational modeling of endothelial electrical resistance, yielding resistance attributable to cell-matrix interaction (Alpha) and the resistance attributable to cell-cell contact (Rb) in human umbilical vein endothelial cells (HUVECs). (A) Absolute endothelial electrical resistance attributable to cell-cell contact (Rb) of wild-type versus Arg-depleted endothelial cells during thrombin stimulation (n = 4). (B) The effects of thrombin on cell-cell contact (%) of wild-type versus Arg-depleted cells. * P < 0.05 in paired t-test (n = 4). (C) The effects of thrombin on cell-matrix interaction (%) of HUVECs treated with imatinib or vector. * P < 0.05 in paired t-test (n = 4). (D) The effects of thrombin on cell-cell contact (%) of HUVECs treated with imatinib or vector. * P < 0.05 in paired t-test (n = 4).
Supplemental Figure S6

Immunofluorescence staining of pY118 Paxillin (green) and VE-cadherin (red) in human umbilical vein endothelial cells for visualization of focal adhesion formation during thrombin stimulation (1U/mL). This figure represents the time course of the images shown in Figure 4C. Scale bars represent 10µm. Representative images of n = 4-6 experiments.
Supplemental Figure S7 Effect of imatinib administration on blood pressure and microvascular perfusion. (A) Systemic arterial blood pressure of male Balb/cJ mice as measured by radio telemetry. Induction of anesthesia and administration of imatinib 20mg/kg (intraperitoneally) were performed according to the protocol used in the Miles assay. n = 3 mice. (B) Representative picture of contrast-enhanced ultrasonography of the right hind limb. This picture shows a cross section of the hind limb at the level of the adductor muscle group. Gas-filled microbubbles were used for contrast enhancement, which show up as green/white (contrast rich) against a dark (contrast poor) background. The red dots and connecting lines show the region of interest used for determination of the microvascular perfusion of the skin. The data analysis of this ultrasonography is presented in Figure 6 of the main manuscript. (C) Cross section of the hind limb at the level of the adductor muscle group, in which red dots and connecting lines show the region of interest used for determination of the microvascular perfusion of muscle. (D) Analysis of microvascular perfusion of the muscle by contrast-enhanced ultrasonography. The graph shows representative contrast replenishment curves after microbubble destruction in a single mouse before and after treatment with imatinib 50mg/kg. The plateau of the replenishment curve represents the microvascular blood volume [A] of the muscle. An extensive explanation of the ultrasonography analysis is provided in the Methods section of the main manuscript. A.U. = Arbitrary Units. (E) The effect of imatinib 50mg/kg on microvascular tissue perfusion of muscle, shown by the microvascular blood volume [A] before (5min) and after (30min) treatment with imatinib. NS = non significant in paired t-test (n = 3 mice).
Legends to Supplemental Video

Supplemental Video 1 Imatinib attenuates thrombin-induced opening of endothelial junctions and promotes formation of focal attachments during thrombin stimulation. Time lapse recordings of human umbilical vein endothelial cells expressing VE-cadherin-GFP and Paxillin-mCherry that are stimulated with thrombin (1U/mL) at \( t = 119 \) minutes. The right movie shows cells that were pretreated with imatinib at \( t = 60 \) minutes, and the left movie shows control cells without imatinib pretreatment. This is a representative recording of 6 movies per condition using endothelial cells from two donors.

Movie information
- Time resolution: 1 minute per frame
- Addition of imatinib/mock: \( t = 60 \) minutes
- Addition of thrombin: \( t = 119 \) minutes
- Green channel: VE-cadherin-GFP
- Red channel: Paxillin-mCherry