Supplementary Data

Dasatinib increases endothelial permeability leading to pleural effusion

Running title: Dasatinib enhances endothelial permeability

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SUPPLEMENTARY MATERIALS AND METHODS

Cell culture

Human pulmonary microvascular endothelial cells (ECs) were isolated and cultured as previously described [9, 44–46]. Briefly, we used lung specimens obtained during lobectomy or pneumonectomy for localized lung cancer in control subjects. The lung specimens from the controls were collected at a distance from the tumor foci. The absence of tumoral infiltration was retrospectively established in all tissue sections by the histopathological analysis. The isolated ECs were strongly positive for acetylated low-density lipoprotein coupled to Alexa 488, von Willebrand factor, CD31, and for Ulex europaeus agglutinin-1 and negative for alpha-smooth muscle actin (α-SMA). The cells were used at passage <5. To suppress Lyn expression, cells were transfected using lipofectamine RNAiMAX with 100nM of Lyn siRNA or with a scrambled sequence (Invitrogen, Cergy-Pontoise, France). The cells were studied within 3 days after transfection. Suppression of Lyn levels was documented 72h after transfection.

Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described before [47]. In short, umbilical cords, obtained from a non-academic hospital, were perfused and incubated (20min, 37°C) with collagenase dissolved in M199 (Biowhittaker/Lonza, Vervier, Belgium). After 30min the umbilical vein was flushed with M199. The perfusate was centrifuged, and the cell pellet was resuspended and seeded on gelatin-coated culture plates. Cells were cultured in M199 medium, 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, USA), 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 on 1% gelatin, at 37°C and 5% CO_2, with a medium change every other day. Cells were cultured up to passage 2, for experiments passage 1–2 cells were used. To suppress Lyn expression, cells were transfected using Dharmafect (Dharmacon, Thermoscientific, Waltham, MA, USA) with 25 nM of Lyn siRNA (Smartpool, Dharmacon) or with a scrambled sequence (Dharmacon/Thermoscientific). The cells

were studied within 2 days after transfection. Cells were treated with dasatinib in indicated concentrations, purchased from Selleckchem (Munich, Germany).

Human pulmonary lymphatic endothelial cells (HPLECs) were purchased from Angioproteomie (Boston, MA, USA). HPLECs were grown in 25cm^2 flasks or 10cm^2 culture disks, coated with rat tail collagenase, and were cultured in EGM-2 culture (Lonza, Verviers, Belgium), according to the providers instruction. Cells were refreshed every other day and split 1:2 when grown confluent. For experiments passage 5–6 cells were used. For splitting, cells were washed once with EDTA and detached with trypsin 0.05% in Hanks Balanced Salt Solution (Lonza). For experiments, HPLECs were seeded in IBIDI 8-well culture slides, coated with gelatin (cross-linked with glutaraldehyde), for immunofluorescence staining or in IBIDI 8W10E ECIS arrays, coated with gelatin for endothelial barrier measurements.

Apoptosis detection

The Annexin V-FITC Assay was used to validate the presence of apoptosis following cell treatment as indicated in media containing 10% FCS. Floating cells were collected with adherent cells harvested by trypsin/EDTA treatment and stained with Annexin V-FITC (BD Biosciences, Pont-de-Claix, France) then analyzed by flow cytometry MACSQuant (Miltenyi Biotec, Paris, France). In each sample, at least 10,000 cells were counted by FACS analysis.

Permeability studies

Barrier function was evaluated by the transfer of HRP or FITC dextran 40kDa across EC monolayers grown in media containing 10% FCS on polycarbonate filters of the Transwell system with 3µm pore as previously described [20, 47]. In addition, electrical cell impedance sensing (ECIS) was used to measure endothelial electrical resistance in confluent monolayers, seeded in 1:1 density (5x10^4 cells/cm^2) on gelatin-coated ECIS arrays, containing 8 wells with 10 gold electrodes/well (Applied Biophysics, Troy, NY, USA).

Western blot and Immunostaining

Cells/tissues were homogenized and sonicated in RIPA buffer containing protease and phosphatase inhibitors and 30µg of protein was used to detect myosin light chain (MLC)-2, phosphorylated (p)MLC-2, nitrotyrosin, GAPDH and β-actin [45, 46, 48–51].

Lipid peroxidation levels were determined using the ALDetect™ Lipid Peroxidation assay kit (Chemicon, Molsheim, France).

Immunohistochemistry and immunocytofluorescent staining for 8-oxo-2'-desoxyguanosine (8-oxo-dG), vascular endothelial (VE)-cadherin, Zonula occludens (ZO)-1 and Tie-2 were performed as previously described [44–46, 51]. Then, sections were saturated with blocking buffer and incubated overnight with specific antibodies, followed by corresponding secondary fluorescent labeled antibodies (Thermo Fisher Scientific, Saint-Aubin, France). Nuclei were labeled using DAPI (Thermo Fisher Scientific). Mounting was done using ProLong Gold antifade reagent (Thermo Fisher Scientific). Images were taken using LSM700 confocal microscope (Zeiss, Marly-le-Roi, France). Rhodamine-phalloidin was from Molecular Probes (Eugene, OR, USA). Then, sections were saturated with blocking buffer and incubated overnight with specific antibodies, followed by corresponding secondary fluorescent labeled antibodies (Thermo Fisher Scientific, Saint-Aubin, France). Nuclei were labeled using DAPI (Thermo Fisher Scientific). Mounting was done using ProLong Gold antifade reagent (Thermo Fisher Scientific). Images were taken using LSM700 confocal microscope (Zeiss). Rhodamine-phalloidin was from Molecular Probes (Eugene, OR, USA). Cryosections of 70µm in thickness were used for the double immunofluorescence staining: LYVE-1 (R&D Systems Europe, Lille, France) and α-SMA (CliniSciences, Nanterre, France) shown in Figure 7.

For immunocytofluorescent studies, HUVECs were seeded on 2cm^2 glass coverslips (5x10^4 cells/cm^2), coated with gelatin. For experiments, culture medium was replaced with 1% HSA/M199 in the presence of vehicle or designated inhibitors. After 90min of preincubation, preincubation medium was replaced by 1% HSA/M199 dasatinib and vehicle/designated inhibitors. Cells were fixated with 4% paraformaldehyde (Sigma Aldrich) and put on ice for 15min. The paraformaldehyde was washed away with phosphate-buffered saline. Subsequently, cells were permeabilized with 0.4% Triton (Sigma Aldrich) in PBS and stained with primary antibodies against VE-cadherin (rabbitXP, Cell Signaling Technologies, 1:400 in 0.1% HSA/PBS) or pSer19-MLC (rabbit, Cell Signaling Technologies, 1:100) overnight at 4°C. After washing, cells were incubated with FITC- or Cy3-labeled secondary antibodies (anti-rabbit, 1:100) (Invitrogen, Paisley, UK) or rhodamine/phalloidine (direct F-actin staining, 1:100) (Invitrogen). Coverslips were mounted with Mowiol Mounting Medium (Sigma Aldrich) or Vectashield mounting

medium, containing DAPI (Vector Laboratories, Burlingham, CA, USA). Imaging was performed with an Axiovert 200 Marianas^{TM} inverted wide-field fluorescence microscope or Nikon 2-photon laser microscope. Imaging was performed at predefined positions on the coverslip. Image analysis was done with ImageJ (National Institutes of Health). Gap quantification was performed with ImageJ as follows. For every image, gap circumference was manually drawn in VE-cadherin/F-actin double immunostainings. Morphometrics for gap circumference was performed, including gap size and gap number.

SUPPLEMENTAL REFERENCES

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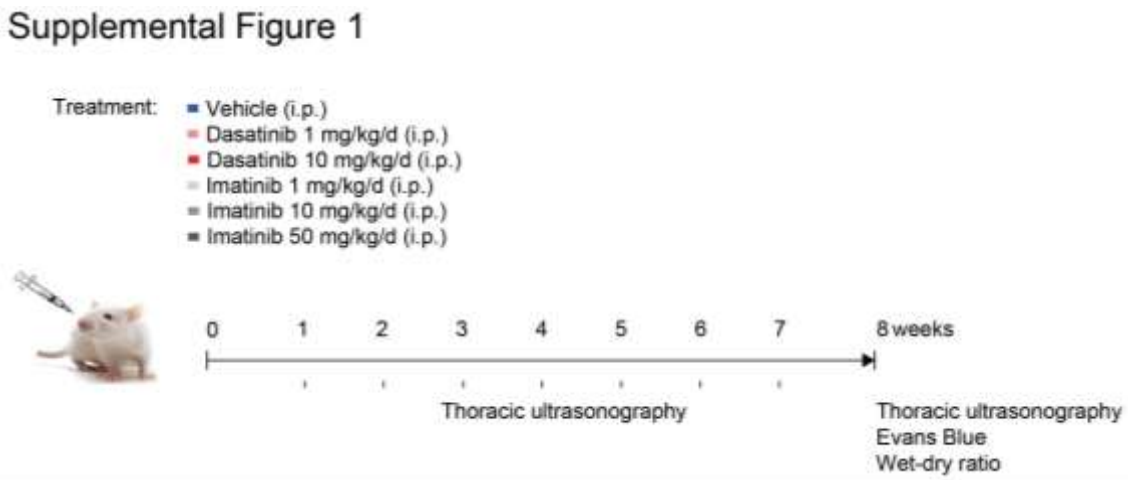
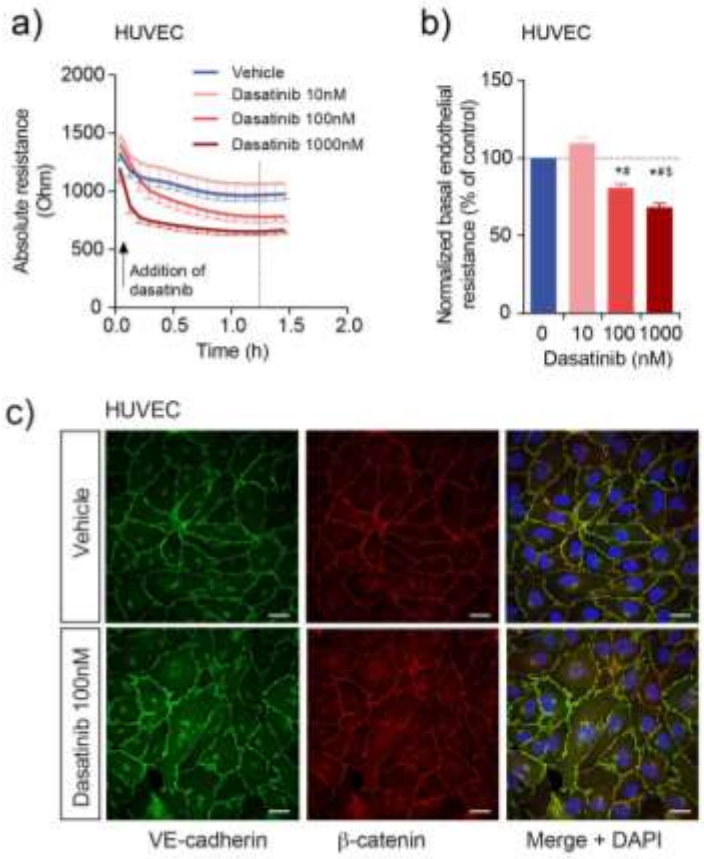
SUPPLEMENTARY FIGURES



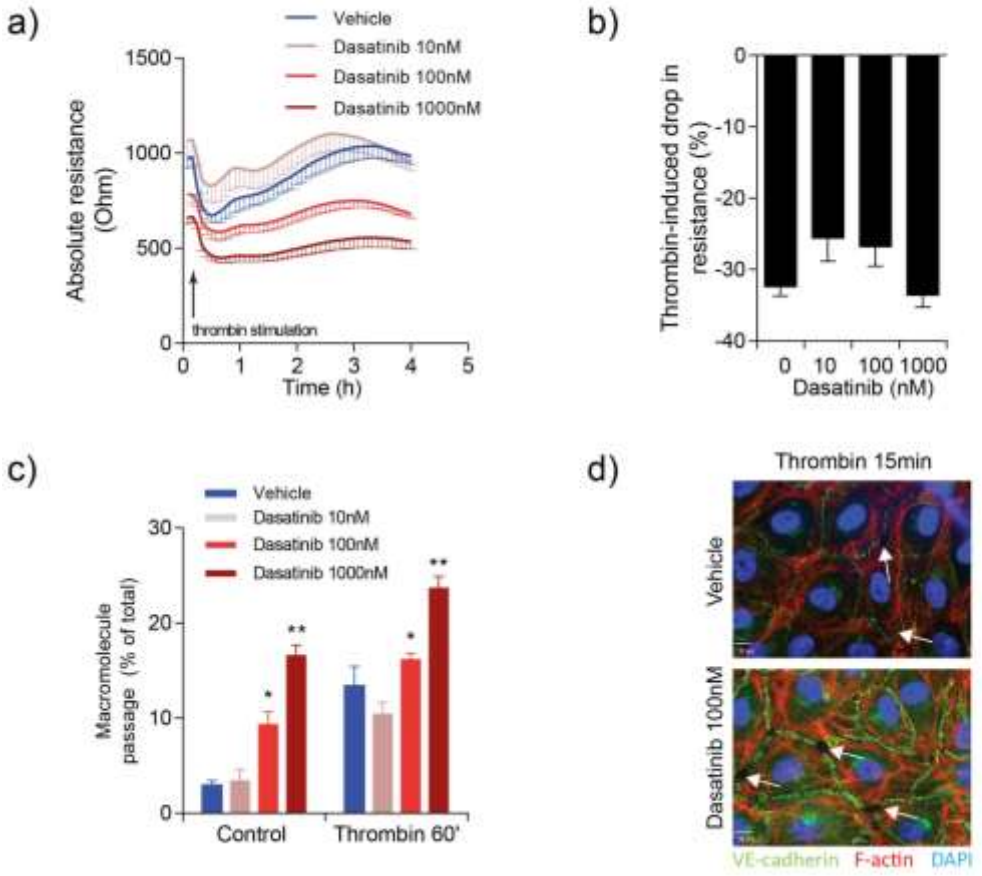
Suppl. Figure 1 – Experimental strategy used to study the effect of dasatinib on pleural effusion in rat.

Supplemental Figure 2



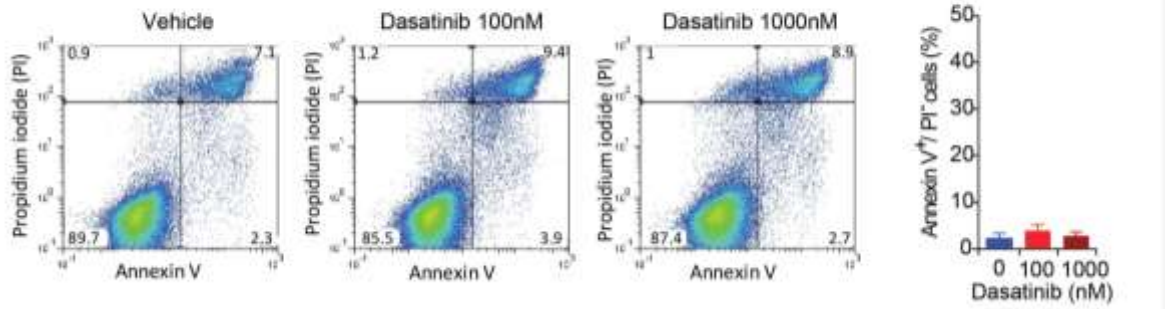
Suppl. Figure 2 – a) Absolute electrical resistance of resting human umbilical vein endothelial cells (HUVEC) monolayers as measured by Electrical Cell-substrate Impedance Sensing after incubation with compounds at indicated concentrations in the presence of 1% human serum albumin in M199. **b)** Resistance normalized to vehicle (0.1% DMSO), 1.2h after addition of indicated compounds. Horizontal lines display the mean±SEM (n=5 donors). c) HUVECs were grown to confluence on glass coverslips, and exposed to dasatinib 100nM or vehicle. After exposure cells were fixed and stained for the adherens junction proteins VE-cadherin, beta-catenin and the nuclear marker DAPI. Scale bar=20µm. Representative images of n=3 independent experiments. *p-value <0.05; $^{\#}$p-value <0.05 versus cells treated with dasatinib 10nM, $^{\$}$p-value <0.05 versus dasatinib 100nM.

Supplemental Figure 3



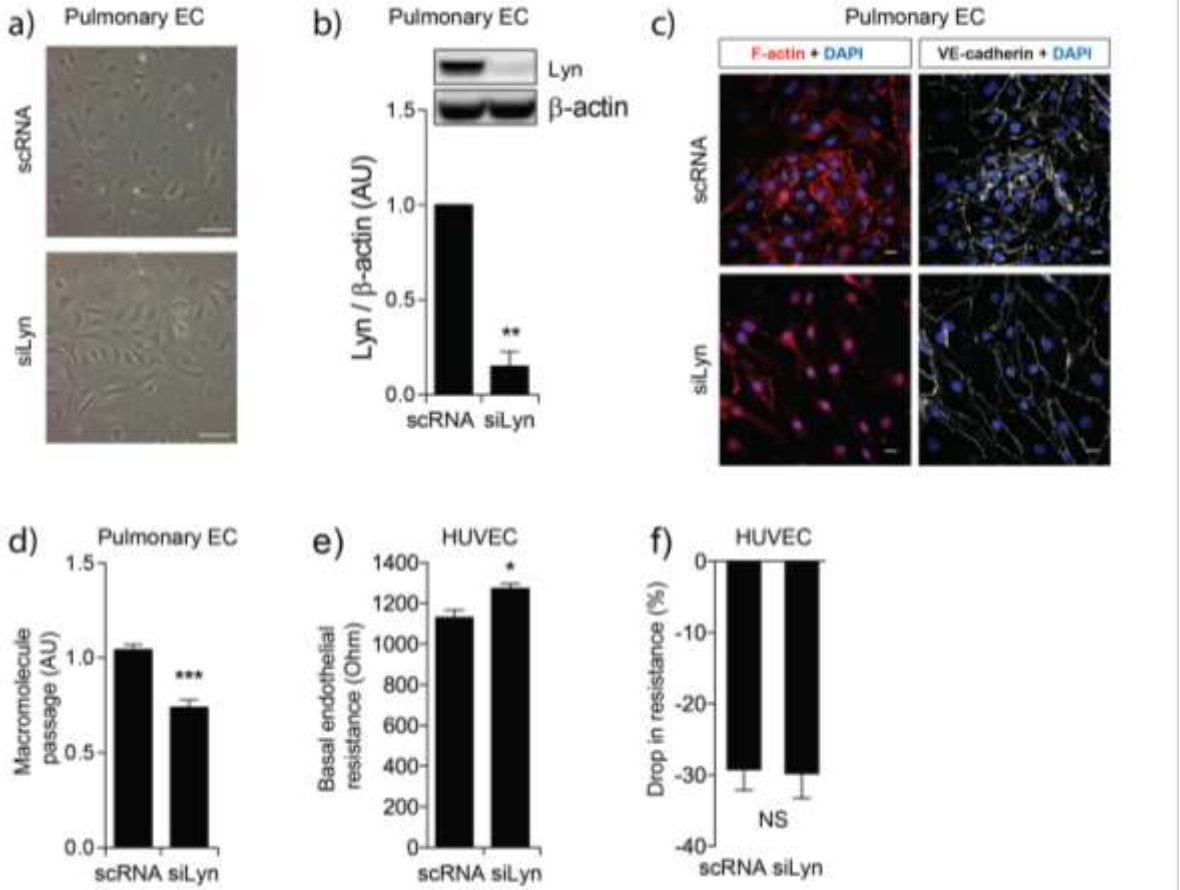
Suppl. Figure 3 – a) HUVECs, grown on gelatin-coated 8W10E ECIS arrays were pre-incubated with serum-depleted medium, supplemented with 1% human serum albumin and dasatinib in indicated concentrations for 1.5h. After stabilization of the monolayer, thrombin (1U/mL) was added. Mean±SEM of n=5 donors. **b)** Thrombin-induced drop in resistance of monolayers. The thrombin-induced drop in resistance was calculated by normalizing resistance to the moment just before thrombin stimulation. Mean±SEM of n=5 donors. **c)** Quantification of macromolecule passage (horse-radish peroxidase or HRP) at different time-points over resting HUVEC pretreated with vehicle or dasatinib in the presence of 1% human serum albumin in M199, and subsequently stimulated with thrombin (1U/mL) or vehicle. Mean±SEM of n=4 donors. **d)** Confocal microscopic analyses and double labeling for vascular endothelial (VE)-cadherin, and direct labeling for F-actin (rhodamine-phalloidin) and the nucleus (DAPI) in resting HUVECs pretreated for 1h to vehicle or 100nM of dasatinib, and subsequently stimulated with thrombin (1U/mL) or vehicle. Arrows indicate intercellular gaps. Scale bar=10 µm. *p-value <0.05; **p-value <0.01 versus vehicle. AU=arbitrary unit. DAPI=4',6-diamidino-2-phenylindole. F-actin=fibrous actin.

Supplemental Figure 4



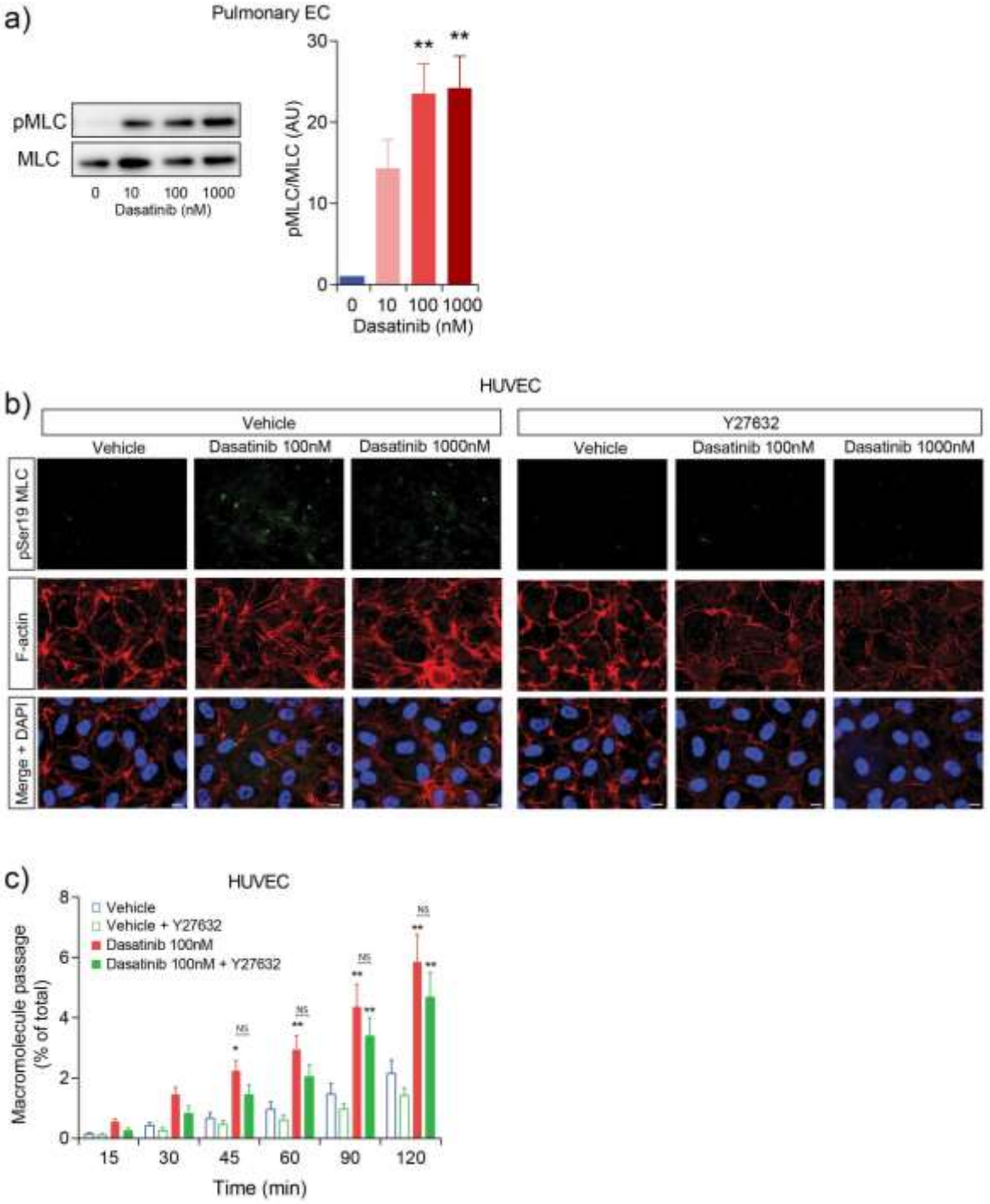
Suppl. Figure 4 – Representative images of the Annexin V-FITC and propidium iodide (PI) dual labeling and quantification of the percentage of Annexin V^+/PI^- human pulmonary ECs treated with vehicle, dasatinib at indicated concentrations in presence of 10% FCS. Mean±SEM of n=4 donors.

Supplemental Figure 5



Suppl. Figure 5 – a) Representative images of human pulmonary endothelial cell (ECs) monolayers (passage <5) 48h after transfection with Lyn siRNA or scrambled sequence (scRNA). Scale bar=10 µm. **b)** Representative Western immunoblots of Lyn and quantification of the Lyn:β-actin ratio. Mean±SEM of n=4 donors. **c)** Confocal microscopy imaging of vascular endothelial (VE)-cadherin and F-actin together with DAPI over resting human pulmonary ECs. Representative images of n=3 donors. Scale bar=20 µm. **d)** Quantification of macromolecule passage of FITC dextran under resting human pulmonary ECs 48h after transfection with Lyn siRNA or scRNA. Mean±SEM of n=4 donors. **e)** Absolute endothelial resistance of resting HUVEC monolayers 48h after transfection with Lyn siRNA or scRNA as measured by Electrical Cell-substrate Impedance Sensing (ECIS) and **f)** quantification of the % drop in electrical resistance upon addition of dasatinib 100nM or vehicle. The dasatinib-induced drop in resistance was calculated by normalizing resistance to the moment just before incubation with dasatinib or vehicle. Mean±SEM of n=4 donors. *p-value <0.05; **p-value <0.001; ***p-value <0.0001 versus cells transfected with scRNA. AU=arbitrary unit. DAPI=4',6-diamidino-2-phenylindole. NS=not significant.

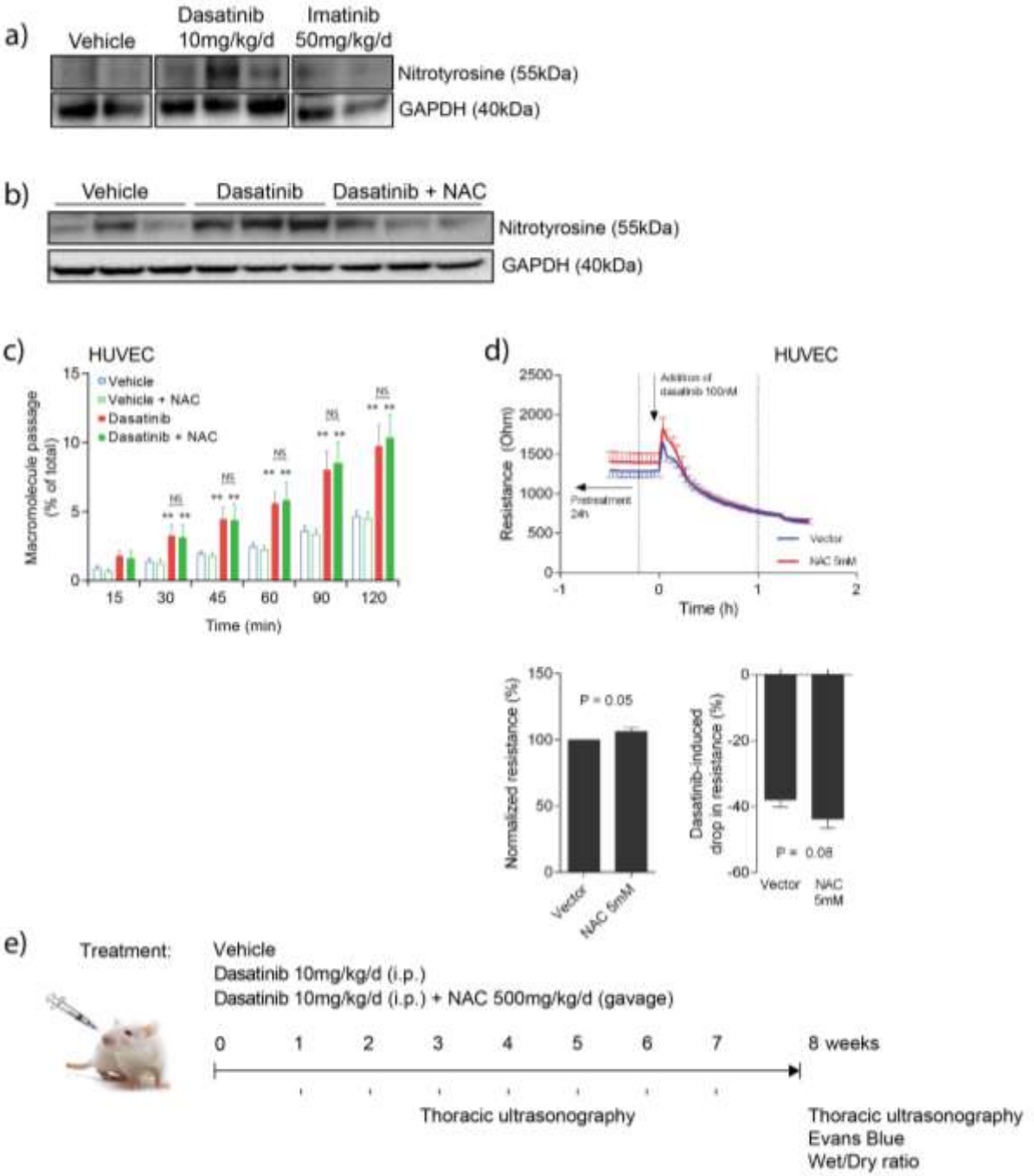
Supplemental Figure 6



Suppl. Figure 6 – a) Representative Western blots and quantification of the phosphorylated myosin light chain (MLC)-2 at Ser_{19}:MLC-2 ratio in resting human pulmonary ECs treated with vehicle, dasatinib at indicated concentrations. **b)** Confocal microscopic analyses and double labeling with antibodies directed against phosphorylated myosin light chain (MLC) at Ser_{19}, or direct labeling of F-actin (rhodamine-phalloidin) and the nucleus (DAPI) in resting HUVEC pretreated or not with Y27632 for 90min and then exposed for 1h to vehicle or dasatinib at indicated concentrations in 1% human serum albumin in M199 with or without a specific ROCK inhibitor Y27632. **c)** Quantification of macromolecule passage (horse-radish peroxidase or HRP) over HUVEC monolayers pretreated or not with Y27632 for 90min and

then exposed to vehicle or dasatinib at different time-points after addition of macromolecule in the presence 1% human serum albumin in M199 to the upper compartment with or without Y27632. *p-value <0.05, ** p-value <0.01, NS=not significant. Scale bar=10 μm in all sections. Horizontal lines display the mean±SEM (n=3–4 donors). AU=arbitrary unit. DAPI=4',6-diamidino-2-phenylindole. F-actin=fibrous actin. MLC=myosin light chain.

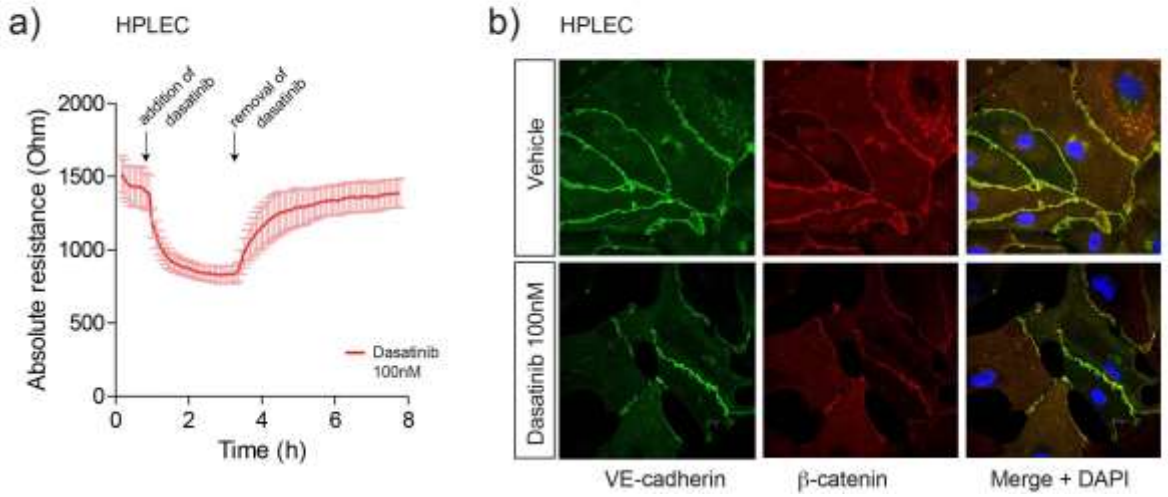
Supplemental Figure 7



Suppl. Figure 7 – a, b) Western blot analysis of nitrotyrosine content in lung lysates from rats treated with indicated compounds. Quantifications are shown in Figure 4a and 4b. **c)** Quantification of macromolecule passage (horse-radish peroxidase or HRP) over resting HUVEC monolayers pretreated or not with the anti-oxidant agent, N-acetyl cysteine (NAC) for 16h and then exposed to vehicle or 1000nM of dasatinib in presence of 1% human serum albumin in M199 with or without NAC for 1.5h. Mean±SEM of n=4 donors. **p-value <0.01 versus vehicle. **d)** Quantification of endothelial electrical resistance of resting HUVEC monolayers after incubation with compounds at indicated concentrations in presence of 10%

FCS with or without NAC for 3 additional hours. Horizontal lines display the mean±SEM (n=4). **e)** Experimental strategy used to study the effect of dasatinib-induced oxidative stress in this rat model of pleural effusion.

Supplemental Figure 8



Suppl. Figure 8 – a) HPLECs were seeded in 8W10E ECIS arrays, and grown to confluence. Longitudinal measurement of electrical HPLECs during exposure to dasatinib 100nM (start at left arrow) and after removal of dasatinib (right arrow) by medium change to culture medium. Mean±SEM of n=4 measurements. **b)** Confluent human pulmonary lymphatic endothelial cells (HPLEC) cultured on glass coverslips and exposed to dasatinib 100nM or vehicle. After exposure cells were fixed and stained for the adherens junction proteins VE-cadherin, beta-catenin and the nuclear marker DAPI.