**Online Data Supplement**

# Bleomycin induces drug efflux in the lungs: A pitfall for pharmacological studies on pulmonary fibrosis

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***Subcutaneous osmotic delivery of bleomycin***

Under isoflurane anesthesia, male C57BL/6J mice of 13 or 24 weeks of age were implanted with subcutaneous osmotic minipumps (ALZET 1007D, DURECT Corporation, Cupertino, CA, USA) through 0.5 cm suprascapular incisions to deliver bleomycin (Hospira, Lake Forest, IL, USA) subcutaneously for 7 days at 0.5 μL/h. Following manufacturer's instructions, pumps were filled with 100 μL of saline (vehicle) or bleomycin solution (100 U/Kg per 84 μL), primed in saline overnight, and implanted for 7 days. Mice were observed through recovery. Pumps were removed on day 7, after delivery of 84 μL of bleomycin or saline (168 hours, 0.5 μL/h, 84 μL). Incisions were closed with wound-clips (Autoclip™, BD Biosciences, San Jose, CA, USA) and sutures (Coated VICRYL, Ethicon, Somerville, NJ, USA), then mice were observed through recovery. A 100 U/kg bleomycin dose was observed to generate progressive fibrosis in 13-week-old mice with day 0 body weights of 26 ±2 grams. The same dose administered to 24-week-old mice with day 0 body weights of 35 ±2 grams was observed to significantly increase mortality and fibrosis as well as dramatically increase efflux pump activity (Supplemental Fig. 1), whereas using mice below a 23 gram body weight caused milder fibrosis at the same dose. Therefore, dose titration of bleomycin in SC-bleo is critical to obtain progressive fibrosis comparable to OP-bleo. PF should be induced by SC-bleo at a 100 U/kg dose in mice with an average body weight of 26 ± 2 grams, providing an optimal dose of bleomycin at 2.6 ± 0.2 U/mouse. Accordingly, mice with average body weight of 26 ± 2 grams were used in both SC-bleo and OP-bleo studies otherwise stated.

***Tissue collection and processing.***

We performed euthanasia of OP-bleo and SC-bleo (at day 7, 14, 28, 42 post-bleomycin delivery) by deep anesthesia with i.p. ketamine/xylazine. Blood was then collected via retro-orbital

puncture, and thoracic cavity was exposed by sectioning through the diaphragm and the rib cage. The left lung and the cranial, middle, and accessory lobes of the right lung were tied off with sutures, removed, and snap-frozen in liquid nitrogen for hydroxyproline measurement or gene expression profiling. The right upper and lower lobes were inflated with 10% neutral buffered formalin via the trachea and immersed in 15 ml formalin for 24 hours, then processed for paraffin-embedding and sectioned (4 μm) onto glass slides for further processing for histology.

***Hydroxyproline measurement by LC-MS/MS.***

Briefly; the left lung was excised, the wet tissue weight was recorded, and the tissue was snap frozen in liquid nitrogen and kept at −80$^{O}$C until use. Lung tissue was homogenized in 600 μL of 0.1 N perchloric acid (PCA). 200 μL of homogenate was aliquoted and prepared for nintedanib measurement as detailed below. 1 mL of 12 N HCL was then added to the remaining 400 μL of lung homogenate, and the homogenate was hydrolyzed at 100° C for 4 hours. Hydrolyzed samples were vortexed and centrifuged at 10,000 *g* for 10 minutes, and 5 μL hydrolysate was diluted 200-fold by the addition of 990 μL of 0.1 N PCA and 5 μL of L-Proline-$^{13}C_5$,$^{15}N$ as internal standard. LC-MS/ MS analyses were conducted on an Agilent 6470 triple quadrupole mass spectrometer (Agilent Technologies) coupled to an Agilent 1260 Infinity II LC system. Hyp was separated using an Intrada Amino Acid column, 50 × 3 mm, 3 μm (Imtakt) at 40° C. Mobile phases consisted of acetonitrile/tetrahydrofuran/25 mM ammonium formate/formic acid = 9:75:16:0.3 (v/v/v/v) (phase A) and acetonitrile/100 mM ammonium formate = 20:80 (v/v) (phase B). Gradient elution (600 μL/min) was initiated and held at 0% B for 3 minutes, followed by a linear increase to 17%

B by 6.5 minutes. This was followed by a step increase to 100% B, which was held until 10 minutes after the gradient was begun, and then by a linear decrease to 0% B by 11 minutes, which was held until 13 minutes after the gradient was begun. The mass spectrometer was set for Agilent jet stream ionization source and operated in positive ion mode. The source parameters were as follows: capillary voltage, 3500V; gas temperature, 300°C; sheath gas temperature, 25°C, sheath gas flow 10 liters/min, gas flow 5 liters/min; nitrogen was used as the nebulizing gas. Collision-induced dissociation (CID) was conducted using nitrogen. Hyp level was analyzed by multiple reaction monitoring. L-Proline-$^{13}C_5$,$^{15}N$ (Sigma, cat#608114) was used as the internal standard. The molecular ion and fragments for Hyp were measured as follows: *m/z* 132.1→86 and 132.1→68 (CID energy: 8 V and 20 V, respectively). Lung levels of Hyp were determined against a standard curve, using trans-4-hydroxy-L-proline as the standard (Sigma-Aldrich). Values are expressed as nmol/left lung.

***Histological and immunohistochemical staining.***

Masson's trichrome staining was performed using Masson's Trichrome Kit (87019) from Thermo Scientific (Waltham, MA, USA) with the following modifications to the manufacturer's protocol: after Bouin's fluid, tissues were rinsed in deionized water for 5 minutes; the Weigert's Iron Hematoxylin step was adjusted to 3 minutes; the Biebrich Scarlet-Acid Fuchsin step was adjusted to 1 minute; and the Aniline Blue step was adjusted to 30 minutes.

Immunohistochemistry was performed as previously described [1]. Murine and human tissue sections were incubated overnight in an optimized blocking solution: 5% blotting-grade milk (Bio-

Rad, Hercules, CA) and 5% horse serum (Vector Laboratories, Burlingame, CA, USA) diluted in double distilled $H_2O$. Rat monoclonal anti-BCRP/ABCG2 antibody (Abcam, Cambridge, MA, USA, Cat. ab24115) was also diluted (1:500 for all mouse tissue; 1:100 for all human tissue) in 5% milk and 5% horse serum solution. The appropriate secondary antibody, anti-rat IgG made in goat (MP-7404, Vector Laboratories), was diluted (1%) in horse serum. Rabbit monoclonal anti-P-Glycoprotein antibody (Abcam, Cat. ab170904) was diluted (1:1000 for all mouse tissue; 1:150 for all human tissue) in 5% milk and 5% horse serum as described above. The appropriate secondary antibody, anti-rabbit IgG made in horse (Cat. MP-7401, Vector Laboratories), was not diluted. Positive immunoreactivity was revealed via chromogenic detection with ImmPACT DAB Peroxidase (HRP) Substrate (SK-4105, Vector Laboratories), then counterstained with hematoxylin-eosin (Gills Formula, Vector Laboratories) for five seconds and covered with a coverslip. The antibodies specificity was tested and optimized by using OP-Bleo administered Mdra/b-Bcrp knock-out mice (Taconic, cat#3998-M) Images were captured with a BX41 bright-field microscope (Olympus, Center Valley, PA, USA). Tile scan images were captured with BX61VS (Olympus, Center Valley, PA, USA) and stitched together using VSI Reader on Fiji software. All images were prepared using Adobe Photoshop CC 2017 to remove non-specimen background images and/or Adobe Illustrator Creative Cloud 2015 (Adobe Systems, San Jose, CA, USA) for formatting.

***Fluorescent in situ hybridization (FISH).***

Fluorescent *in situ* hybridization (FISH) was performed using the RNAscope® Multiplex Fluorescent Kit v2 (Advanced Cell Diagnostics (ACD), Newark, CA, USA, Cat. 323110) and RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual on formalin fixed paraffin

embedded (FFPE) lung tissue from mice that were 14 days post OP-Bleo [2]. We chose the 14-day time point because this was the only time point that had a significantly higher expression of both Abcg2 and Abcb1b *in vivo*. Images were collected using the Zeiss LSM700 confocal microscope.

Lung tissue was fixed in 10% neutral buffered formalin (Sigma) for 24 hours and then embedded in paraffin (Fisher Scientific). FISH was performed on 4-micron thick FFPE lung tissue sections. Sectioned lung tissue was baked at 60º C for 60 min and then deparaffinized in two 5 min washes of xylene followed by two 5 min washes of 100% ethanol. Endogenous peroxidases were removed from the lung tissue by using RNAscope® Hydrogen Peroxide (ACD, Cat. 322335) for 10 min at room temperature. Antigen retrieval was then performed by boiling lung tissue in RNAscope® Target Retrieval (ACD, Cat. 322000) at 99-100º C for 15 min. Slides were stored with desiccants at room temperature overnight.

RNAscope® Protease Plus (ACD, Cat. 322331) was then used to digest the lung tissue at 40º C for 30 min. RNAscope® ISH probes from ACD were used at recommended dilutions and incubated on lung tissue at 40º C for 120 min. The probes used from ACD were *Col1a1* in channel 2 (Cat. 319371-C2), *Cspg4* in channel 2 (Cat. 404131-C2), *Pecam1* in channel 2 (Cat. 316721-C2), *Sftpc* in channel 2 (Cat. 314101-C2), *Cd68* in channel 2 (Cat. 316611-C2), *Abcb1b* in channel 1 (Cat. 422191), and *Abcg2* in channel 3 (Cat. 510101-C3). The fluorophores used to visualize the RNAscope® ISH probes were cyanine 3 and 5 from the TSA Cyanine 3 & 5, TMR, Fluoresein Evaluation Kit (Perkin Elmer, Waltham, MA, USA; Cat. NEL760001KT). Cyanine 3 and 5 were diluted in RNAscope® LS Multiplex TSA Buffer (ACD, Cat. 322810). The fluorophore dilutions

for each RNAscope® ISH probe were *Abcg2*-Cyanine 3 (1:750), *Abcb1b*-Cyanine 3 (1:750), *Cd68*-Cyanine 5 (1:750), *Pecam1*-Cyanine 5 (1:750), *Sftpc*-Cyanine 5 (1:15000), *Col1a1*-Cyanine 5 (1:7500), and *Cspg4*-Cyanine 5 (1:750). The positive control probes for determining lung tissue RNA quality were *Polr2a* in channel 1 and *Ubc* in channel 3 from the RNAscope® 3-Plex Positive Control Probes (ACD, Cat. 320881). Fluorophore dilutions for positive control probes were *Polr2a*-Cyanine 3 (1:750) and *Ubc*-Cyanine 5 (1:750). The negative control probe for determining nonspecific background staining was bacterial gene *dapB* in channels 1 and 3 from the RNAscope® 3-Plex Negative Control Probe (ACD, Cat. 320871). The fluorophore concentrations for the negative control probe was *dapB*-channel 1-Cyanine 3 (1:750) and *dapB*-channel 3Cyanine 5 (1:750). All washes, AMP steps, and blocking steps were performed according to the RNAscope® Multiplex Fluorescent Reagent Kit v2 User Manual.

***Lung function measurements***

Lung function measurements were performed at the end of the study as a terminal procedure. Mice were anesthetized by intraperitoneal (IP) injection of Ketamine/Xylazine, then an 18-gauge metal cannula was inserted into the trachea by small incision. Pancuronium was then administered by IP injection (0.8 mg/kg) to induce paralysis before connecting mice to flexiVent and starting ventilation. Pressure-volume (PV) curve, airway resistance, tissue damping (G), tissue elastance (H), and forced expiratory volume per 0.1 seconds (FEV 0.1) parameters were collected. Then mouse tissue collection was conducted.

***RNA isolation and reverse-transcription quantitative polymerase chain reaction (RT-qPCR).***

Total RNA was isolated using RNeasy® Lipid Tissue Mini Kit from Qiagen (Hilden, Germany), according to the manufacturer's protocol. 1 μg of RNA was reverse-transcribed using an iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) according to the protocol. The differential expression of all target genes in this study was measured using sequence-specific QuantiTect® primers (Qiagen) and SYBR® Green Master Mix (Thermo Scientific) on a StepOnePlus 7500 Real-Time PCR Instrument (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated based on the standard $\Delta\Delta C_t$ method using *Tbp* (Qiagen, QT00198443) expression as references for experiments on mouse tissue.

***Nintedanib treatment and tissue measurement.***

Nintedanib (BIBF 1120) esylate (MedChemExpress, Monmouth Junction, NJ) was administered by oral gavage once daily at 3 mg/ml concentrations to achieve a 30 mg/kg dose, one hour prior to euthanasia. Vehicle was used at ratio of DMSO/Tween 80/saline (1:1:18).

Lung and plasma were extracted as described previously [3]. 200 μL of left lung homogenate described above and 30 μL of plasma were used. Nintedanib levels were determined by LC-MS/MS using an Agilent 6470 triple quadrupole mass spectrometer (Agilent Technologies) coupled to an Agilent 1260 Infinity II LC system (Agilent Technologies). Chromatographic and mass spectrometer conditions were set as following. Nintedanib was separated using Agilent Poroshell 120, EC-C18 2.7 μm column maintained at $34^0$C. Gradient elution mobile phases consisted of A (0.1% formic acid in $H_2O$) and B (0.1% formic acid in MeOH). Gradient elution (350 μL/min) was initiated at 10% B, followed by a linear increase to 50% B by 0.5 min, which was followed by another linear increase to 85% B by 3 min and maintained until 14 min, then

increased linearly to 100% B at 18 min and maintained until 38 min. Agilent jet stream ion source operated in positive ion mode. The source parameters were as follows: capillary voltage, 3500V; gas temperature, 300°C; sheath gas temperature, 25°C, sheath gas flow 10 liters/min, gas flow 5 liters/min; nitrogen as the nebulizing gas. Collision-induced dissociation (CID) was performed using nitrogen. Levels of nintedanib were analyzed by multiple reactions. The molecular ion and fragments for each compound were measured as follows: *m/z* 540.3→113 and 540.3→70.2 for nintedanib (CID energy: 28 V and 72 V, respectively). The amounts of nintedanib in the samples were determined against standard curves. Values are expressed as ng/g or ng/ml in wet tissue weight or plasma volume, respectively.

References


1. Cinar, R., et al., *Hybrid inhibitor of peripheral cannabinoid-1 receptors and inducible nitric oxide synthase mitigates liver fibrosis.* JCI Insight, 2016. **1**(11).
2. Wang, F., et al., *RNAscope: A Novel in Situ RNA Analysis Platform for Formalin-Fixed, Paraffin-Embedded Tissues.* The Journal of Molecular Diagnostics, 2012. **14**(1): p. 22-29.
3. Mukhopadhyay, B., et al., *Hyperactivation of anandamide synthesis and regulation of cell-cycle progression via cannabinoid type 1 (CB1) receptors in the regenerating liver.* Proc Natl Acad Sci U S A, 2011. **108**(15): p. 6323-8.

**Supplementary figure legends**

**Figure E1**. P-gp and BCRP protein levels were elevated at 14 days post-bleo then remain elevated by 42 days post-bleo in bleomycin-induced PF in mice. (**A**) Mouse lung sections were stained with Masson's trichrome staining. (**B**) P-gp protein levels and (**C**) BCRP protein levels were showed in immunohistochemistry experiments in OP-bleo and in SC-bleo. n=4 mice per control group and 5 mice per OP-bleo and SC-bleo groups.

**Figure E2**. 0.3 U OP-bleo still induce PgP and BCRP overexpression that compromise target exposure of nintedanib. (**A**) Masson trichrome stainings, (**B**) hydroxyproline levels, (**C**) protein expressions of P-gp and BCRP, and (**D**) gene expressions of Abcb1a, Abcb1b and Abcg2 in mice lung. (**E**) Levels of nintedanib in lung and serum one hour after single oral dosing at 30 mg/kg. n=4 mice per control group and 5 mice per OP-bleo and SC-bleo groups.

**Figure E3.** Op-bleo (1U/kg) more significantly induced P-gp in male mice lung than female. (**A**) Body weight changes from the initial weights as of 100%. (**B**) Hydroxyproline content in lung. (**C**) P-gp immunostaining in lung of either control or OP-bleo instilled mice after 7 days post-bleomycin. n=3 mice per control group and 4 mice per OP-bleo groups.

**Figure E4.** Age and dose dependent effect of bleomycin in SC-bleo-induced PF model in mice. (**A**) Hydroxyproline content in lung. (**B**) Body weight changes from the initial weights as of 100%. (**C**) P-gp and BCRP immunostainings.

**Figure E5.** SC-bleo increased P-gp protein expression in small intestine. P-gp immunostaining in small intestine of normal and SC-bleo (100 U/kg) challenged mice at 14 days after the initial bleomycin exposure. n=3 mice per group.

Figure E1

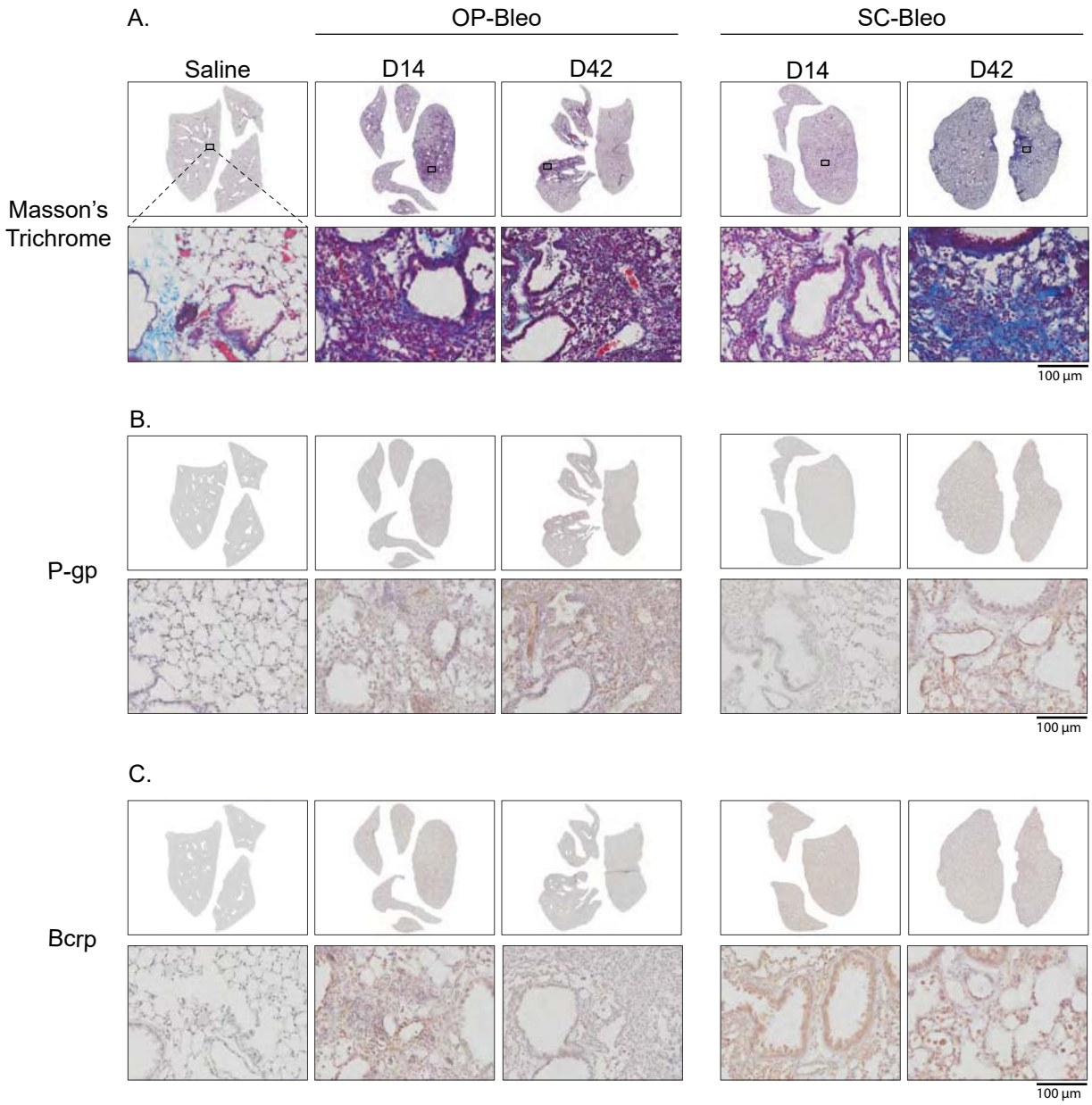
A.
OP-Bleo
SC-Bleo
Saline
D14
D42
D14
D42
Masson's Trichrome
100 µm
B.
P-gp
100 µm
C.
Bcrp
100 µm

Figure E2

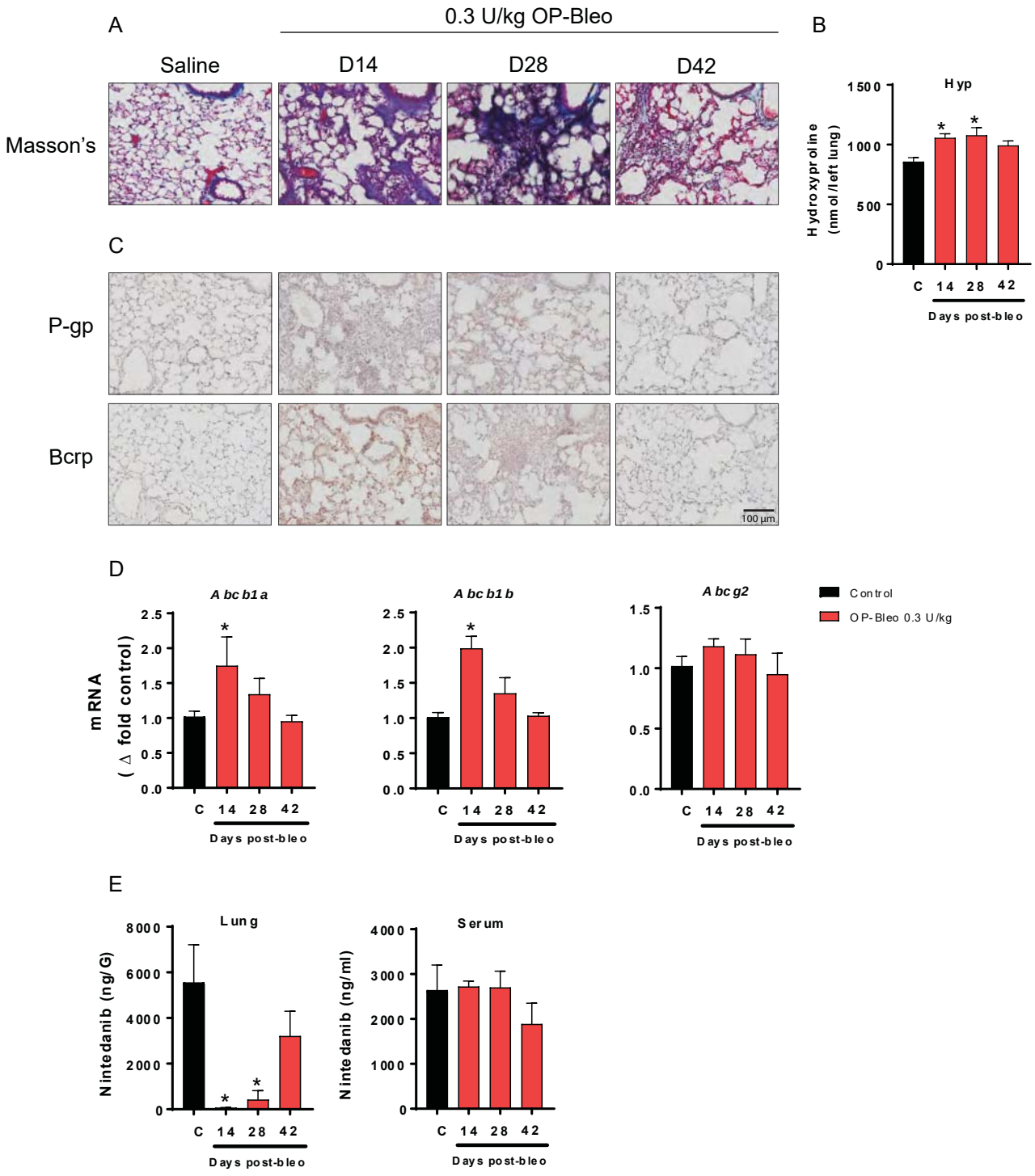
A
0.3 U/kg OP-Bleo
Saline
D14
D28
D42
Masson's
B
Hyp
Hydroxyproline (nmol/left lung)
1500
1000
500
0
C
14
28
42
Days post-bleo
C
P-gp
Bcrp
100 µm
D
Abcb1a
Abcb1b
Abcg2
mRNA (Δ fold control)
2.5
2.0
1.5
1.0
0.5
0.0
Control
OP-Bleo 0.3 U/kg
E
Lung
Serum
Nintedanib (ng/G)
Nintedanib (ng/ml)
8000
6000
4000
2000
0
3000
1000

Figure E3

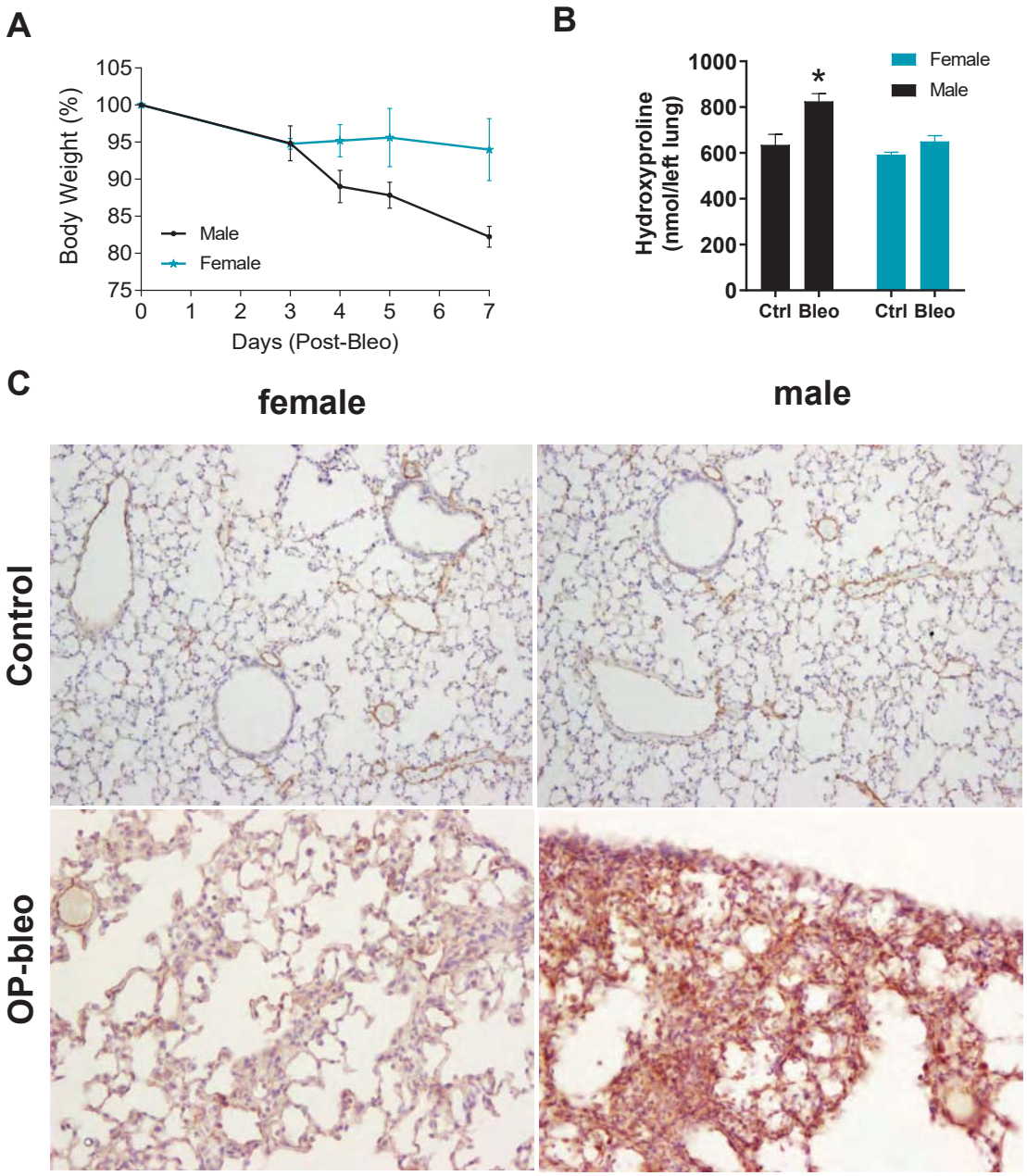
A
Body Weight (%)
105
100
95
90
85
80
75
0 1 2 3 4 5 6 7
Days (Post-Bleo)
Male
Female
B
Hydroxyproline (nmol/left lung)
1000
800
600
400
200
0
*
Female
Male
Ctrl Bleo
Ctrl Bleo
C
female
male
Control
OP-bleo

Figure E4

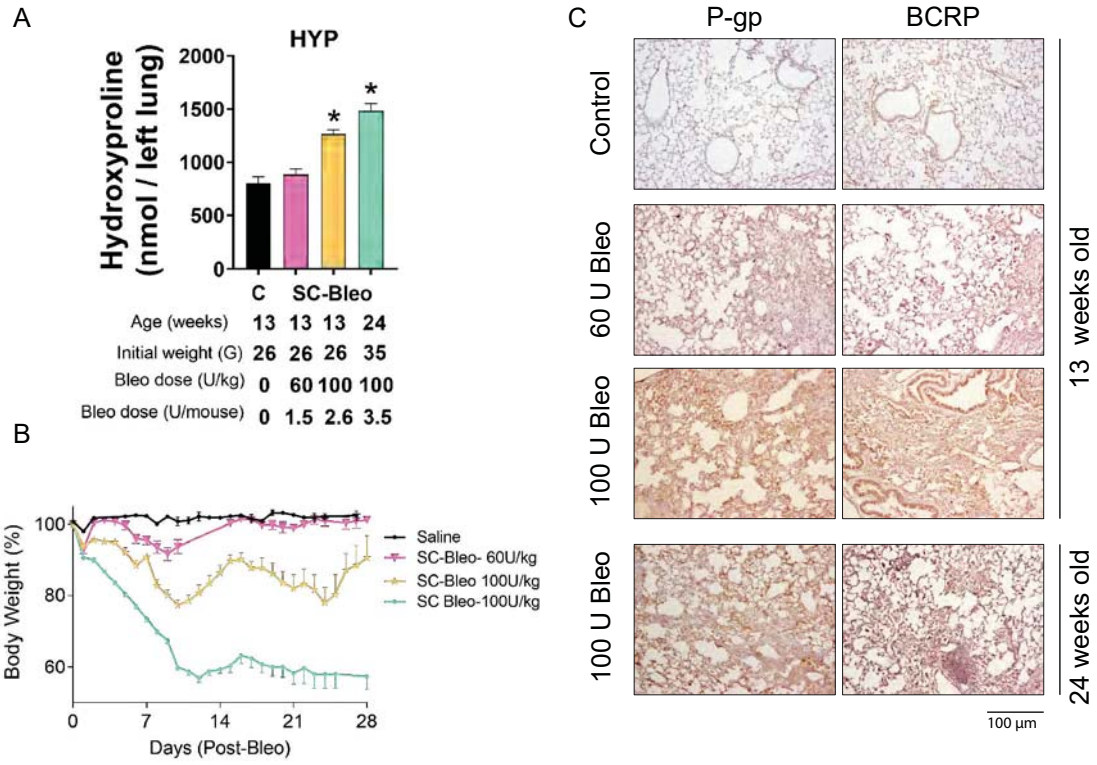
A
HYP
Hydroxyproline (nmol / left lung)
2000
1500
1000
500
0
*
*
C
SC-Bleo
Age (weeks) 13 13 13 24
Initial weight (G) 26 26 26 35
Bleo dose (U/kg) 0 60 100 100
Bleo dose (U/mouse) 0 1.5 2.6 3.5
B
Body Weight (%)
100
80
60
0
7
14
21
28
Days (Post-Bleo)
Saline
SC-Bleo- 60U/kg
SC-Bleo 100U/kg
SC Bleo-100U/kg
C
P-gp
BCRP
Control
60 U Bleo
100 U Bleo
100 U Bleo
13 weeks old
24 weeks old
100 µm

Figure E5