Supplementary Material to Zhao et al. “A novel hirudin derivative inhibiting thrombin without bleeding for subcutaneous injection” (Thromb Haemost 2017; 117.1)

Suppl. Methods

Immunohistochemistry

Paraffin embedded lung tissues were cut into 10 µm thick sections. After being posted on the slide, tissues were treated with xylene and deparaffinized. The sections were dehydrated in graded alcohol. After sections were rinsed in phosphate-buffered saline (PBS, pH 7.4), the sections were incubated with 3% hydrogen peroxide for 30 min. Sections were incubated with 5% goat serum for 30 min at RT to block non-specific binding sites, then sections were incubated with an anti-CD41 antibody (Abcam) and thrombin F-1 (Santa Cruz Biotechnology INC). Incubations with the primary antibody were conducted overnight at 4 °C, and then sections were washed thrice with PBS and incubated with an HRP polymer conjugate (Invitrogen Corp.) for 1 h at RT. Sections were subsequently washed thrice with PBS and incubated with streptavidin peroxidase for 10 minutes. The same sections were washed and reacted with 3,3’-diaminobenzidine (DAB; DAKO Corp.) for 10 minutes at room temperature and counter-stained with Mayer's hematoxylin. Then, the sections were observed under a light microscope (Nikon, Tokyo, Japan).

Immunofluorescence and confocal microscopy

The lungs were fixed with 4% formaldehyde solution for 24 h and were dehydrated in 15% sucrose and 30% sucrose. The sample was embedded in Tissue-Tek II O.C.T. Mountant (Miles Laboratories, Inc., Naperville, IL) prior to freezing in liquid nitrogen. The embedded tissues were cut into 10 µm thick sections. Sections were washed with PBS and incubated with 5% goat serum for 30 min at RT to block non-specific binding. Sections were incubated with anti-CD41 antibody (Abcam) and thrombin F-1, (Santa Cruz Biotechnology INC). Incubations with the primary antibody were conducted overnight at 4°C. Sections were then washed thrice with PBS and incubated with Alexa 594-conjugated IgG antibody (Bethyl Laboratories, Inc) and FITC-conjugated IgG antibody (Bethyl Laboratories, Inc) for 1 hour at RT and viewed with a confocal microscope (Olympus).
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Suppl. Figure 1. Expression, Purification and Characterization of DTIP. A: Mass spectrometry of purified DTIP showing its molecular weight was 3923 Da, while molecular weight of the sodium adducts was 3945 Da. B: HPLC analysis of purified DTIP showing up to 95% purity.
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Suppl. Figure 2. Effect of DTIP in rats. Sprague Dawley (SD) rats (300 ± 30 g, male) were anesthetized with chloral hydrate (300 mg/kg), and given bivalirudin (1.0 mg/kg; iv, n=10; sc, n=10) and DTIP (1.0 mg/kg; iv, n=10; sc, n=10) via intravenous (iv) and subcutaneous (sc). Blood samples were collected 0.5-1 h after treatment from carotid artery. aPTT (A), PT (B), TT (C) and clotting time (D) were were measured. (1-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001, NS, not significant. Data are expressed as mean ± SD.)
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Suppl. Figure 3. Epinephrine and collagen-induced lethal pulmonary thromboembolism. Saline, bivalirudin (1.0 mg/kg, iv, sc) and different concentration of DTIP (iv and sc) were treated. Within 30 min, all of mice were injected with a mixture of recombinant collagen (4 mg/kg) and epinephrine (0.3 mg/kg) through the tail vein. A: The survival rates were recorded. Immunohistochemistry (B) and immunofluorescence (C) of the lungs of mice. (Compared with saline group by Fisher's exact test. * P < 0.05, ** P < 0.01. *** P < 0.001, NS, not significant.)
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Suppl. Figure 4. FeCl\textsubscript{3}-injured thrombus formation in mouse mesenteric arterioles. Saline, bivalirudin (1.0 mg/kg, iv, sc) and different concentration of DTIP (iv and sc) was injected 30 minutes before 10% FeCl\textsubscript{3} injury of the mesenteric arteriole and observed under a dissecting microscope. (Compared with saline by one-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001. Data are expressed as mean ± SD.)
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Suppl. Figure 5. Bleeding effect of DTIP in mice. The distal 5.0 mm of the tail was transected and immersed in saline kept at 37°C. Bleeding time and blood loss were measured as described in Materials and methods. Bivalirudin (1.0 mg/kg, iv, n=11) and DTIP (1.0 mg/kg; iv, n=8; sc, n=10) were given. Each dot represents the bleeding time and blood loss volume measured in individual mouse, mean ± SD is also shown. (Compared with saline by one-way ANOVA. * P < 0.05, ** P < 0.01, *** P < 0.001, NS, not significant. Data are expressed as mean ± SD.)
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Suppl. Figure 6. Molecular docking of thrombin and its direct inhibitor. A: Thrombin docked with r-RGD-hirudin. B: Thrombin docked with DTIP.