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Supplementary figures

Inhibiting sialidase-induced TGF-β1 activation attenuates pulmonary fibrosis in mice

Tejas R. Karhadkar [1], Thomas D. Meek [2], and Richard H. Gomer [1]

[1] Department of Biology,

[2] Department of Biochemistry and Biophysics,

Texas A&M University

301 Old Main Drive, College Station, Texas, 77843-3474 USA

Corresponding author:

Richard H Gomer

Department of Biology

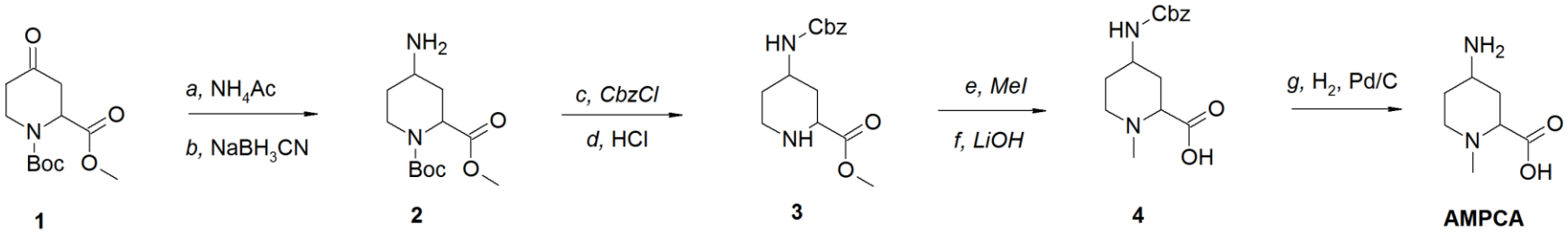
Texas A&M University

301 Old Main Drive

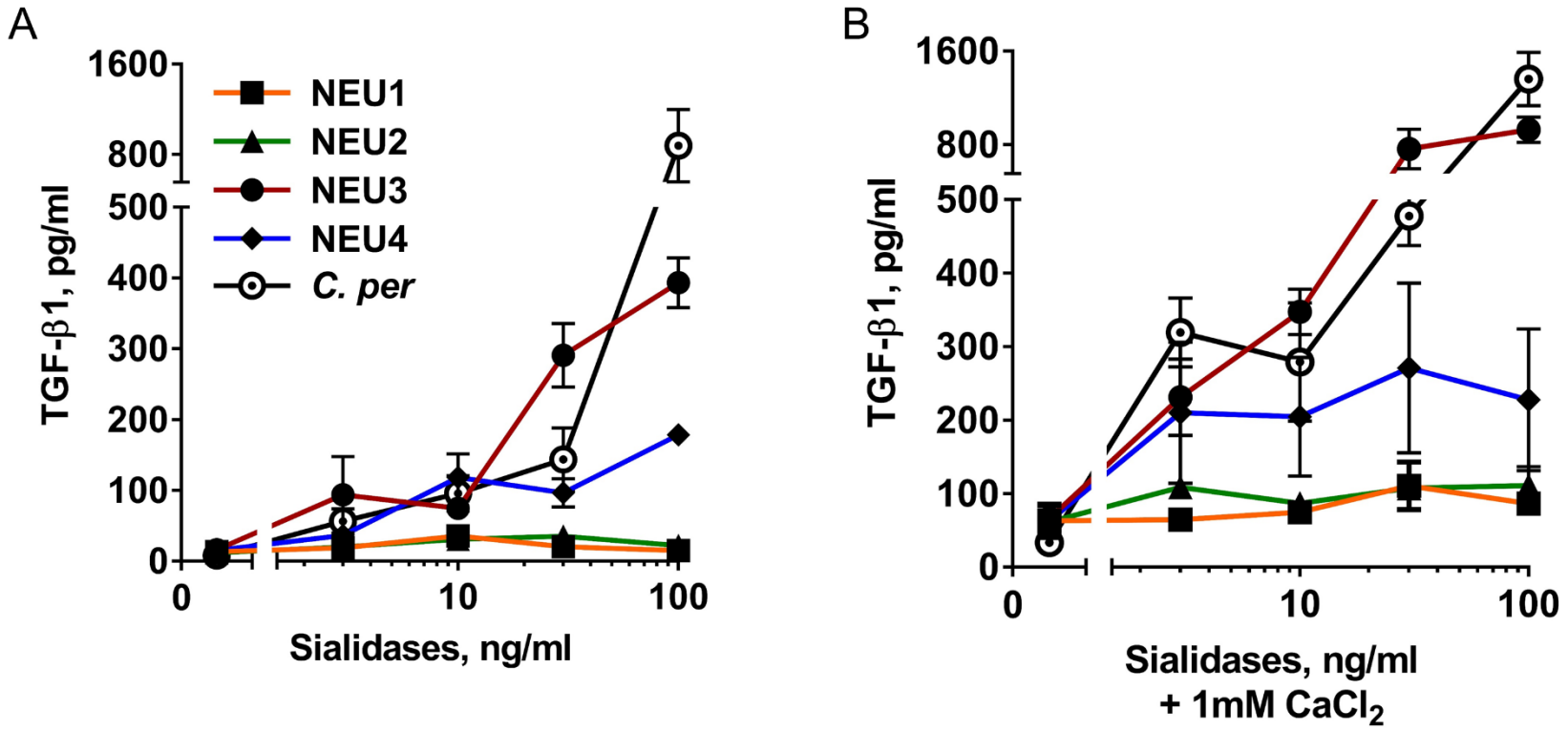
College Station, TX 77843-3474 USA

Phone: 979-458-5745. Fax: 979-845-2891. email: rgomer@tamu.edu

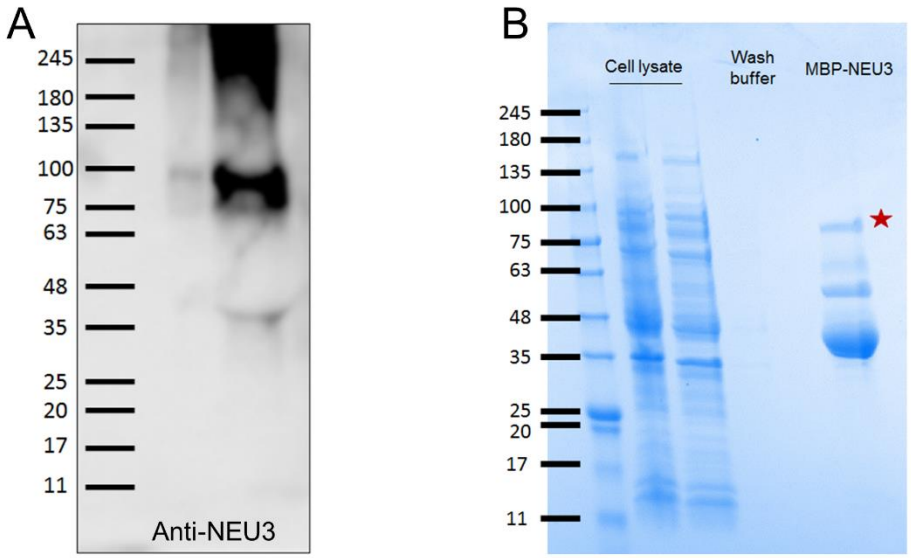
Supplementary figures and supplementary figure legends



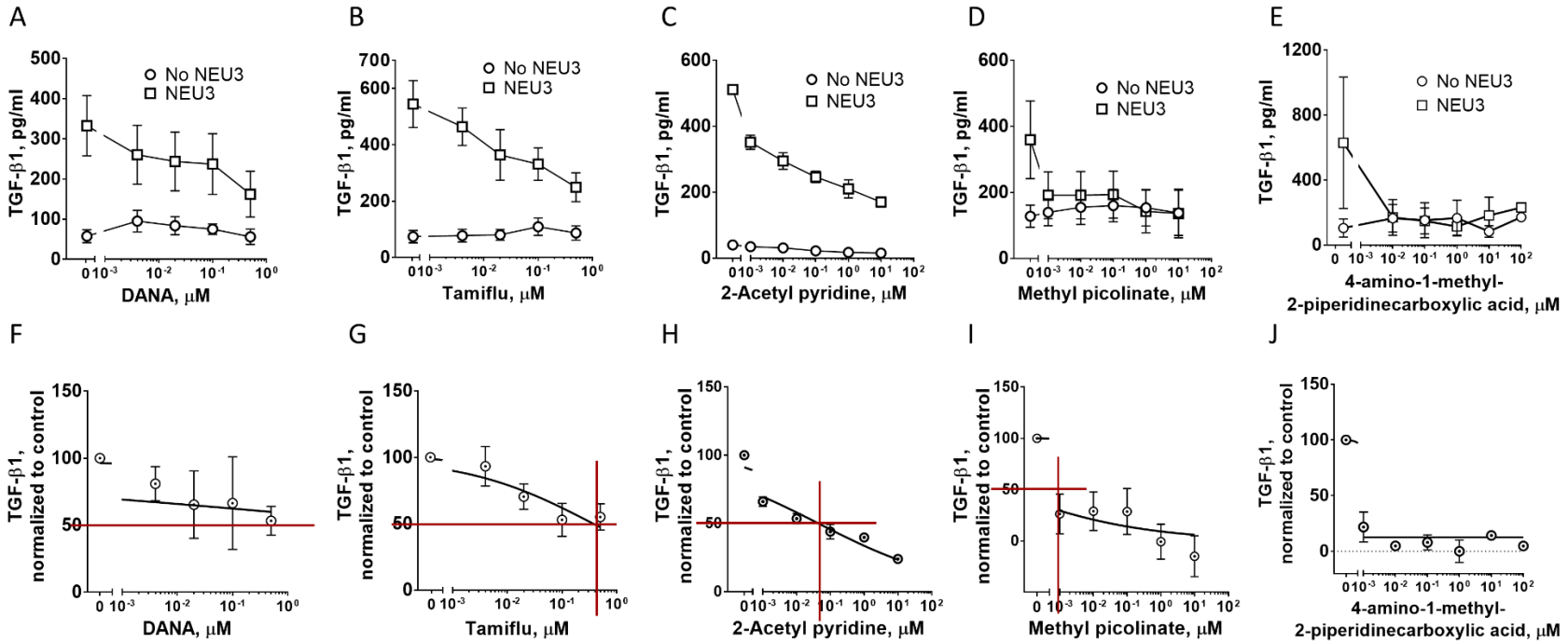
Supplementary Figure 1. Synthetic scheme for 4-amino-1-methylpiperidine-2-carboxylic acid (AMPCA). Ammonium acetate was reacted with the 4-keto group of 1-(*tert*-butyl) 2-methyl 4-oxopiperidine-1,2-dicarboxylate (**1**) to form an imine (*a*), followed by reduction (*b*) with sodium cyanoborohydride to afford the amine **2**. The installed amine was then protected by reaction with benzyloxycarbonyl chloride (*c*). The *t*-Boc group was removed by treatment with HCl (*d*), The piperidine nitrogen was then methylated in base by methyl iodide (*e*), and the methyl ester was then hydrolyzed by treatment with LiOH (*f*). The Cbz protecting group was then removed by hydrogenation catalyzed by palladium on carbon (*g*) to afford AMPCA.



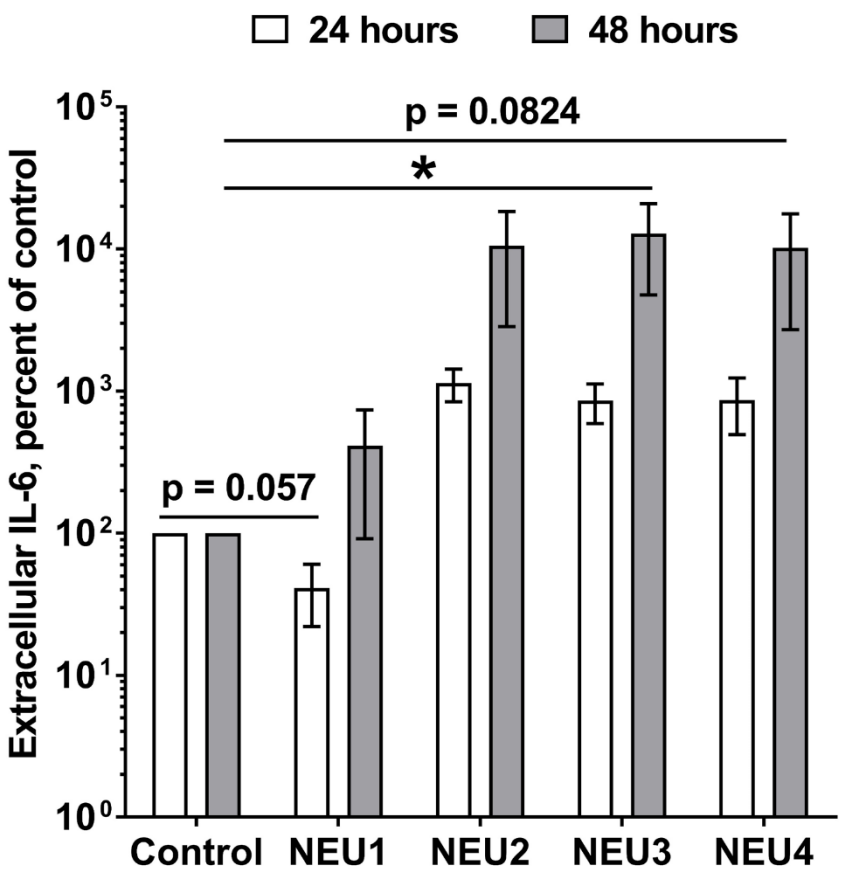
Supplementary Figure 2. Ability of sialidases to release active TGF-β1. An ELISA assay, specific for active TGF-β1, was performed on recombinant human latent-TGF-β1 (rhL-TGF-β1) treated with the indicated concentrations of NEU 1 – 4 or *C. perfringens* (*C. per*) neuraminidase in **(A)** the absence or **(B)** the presence of 1 mM $CaCl_2$. Values are mean ± SEM, n = 3.



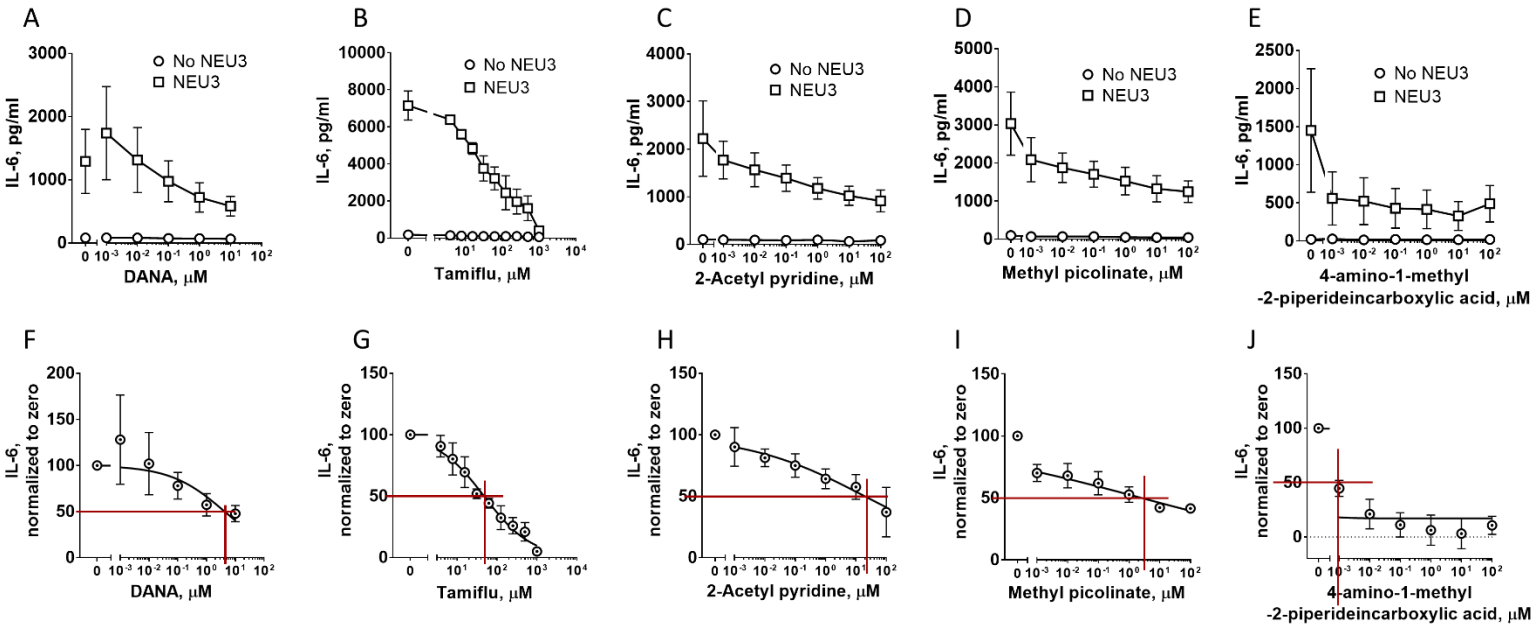
Supplementary Figure 3. Recombinant human NEU3 purified from bacteria. (A) A western blot of purified MBP-NEU3 was stained with anti-NEU3 antibodies, showing a band at the expected molecular mass of ~93 kDa, (**B)** A gel of the indicated fractions was stained with Coomassie. The desired MBP-NEU3 is marked with a star (★).



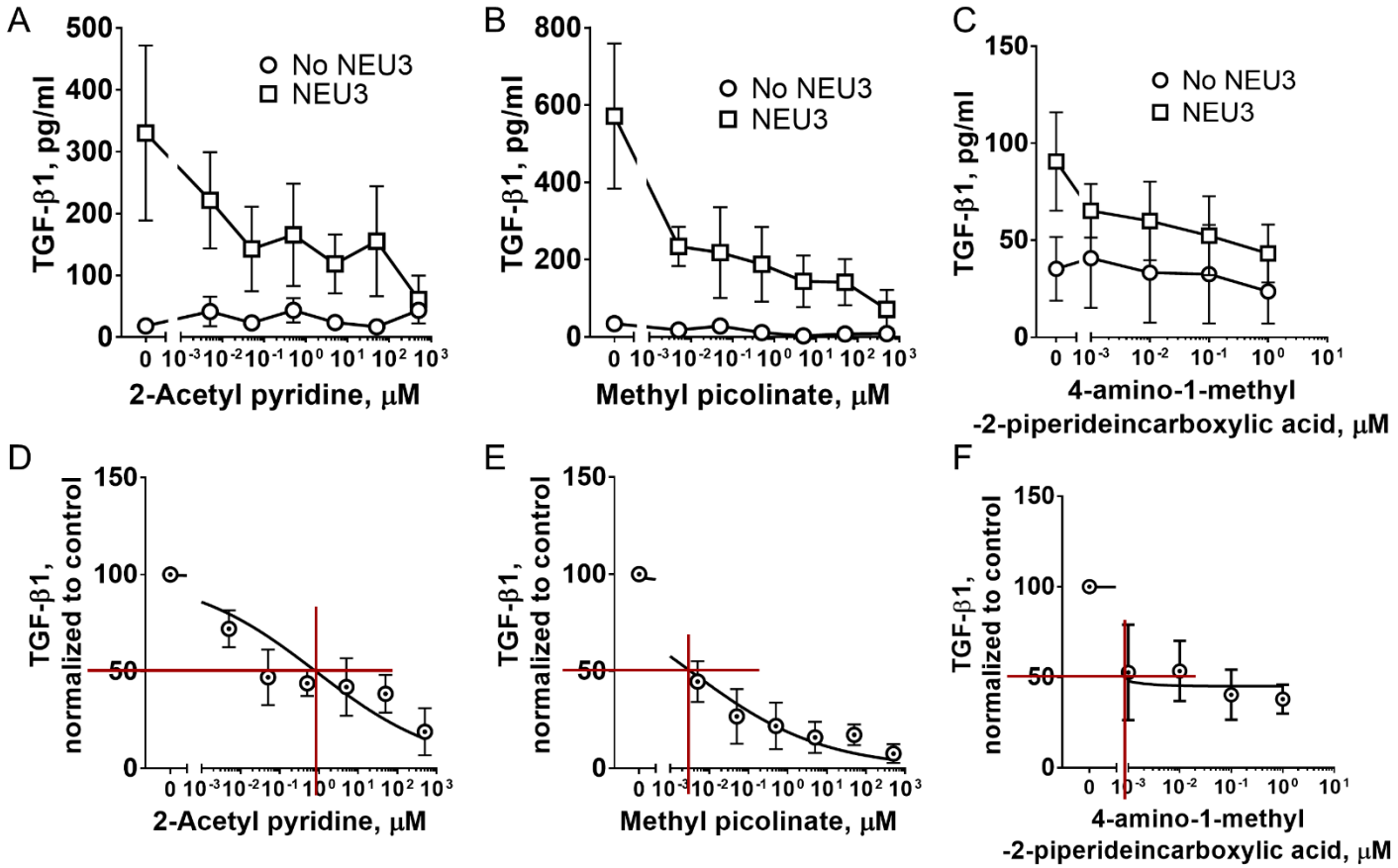
Supplementary Figure 4. Inhibition of recombinant human NEU3-catalyzed release of active TGF-β1 by compounds from Table 1. Compounds labelled on the X-axis of graphs **(A)** to **(E)** were incubated at the indicated concentrations with and without recombinant human NEU3 in PBS pH 6.9. rhL-TGF-β1 was added as substrate, and the release of active TGF-β1 was quantified by an ELISA kit. Values are mean ± SEM, n ≥ 3. The NEU3-catalyzed release of active TGF-β1 was determined by subtracting active TGF-β1 values in the absence of NEU3 from active TGF-β1 values in the presence of NEU3 in the data from (A to E). The baseline corrected values were normalized to the percent of active TGF-β1 in the absence of the compound (0 μM of inhibitor). **(F)** – **(G)** IC_{50} values (red lines) were determined from the normalized values by non-linear regression curve fitting. Values are mean ± SEM, n ≥ 3.



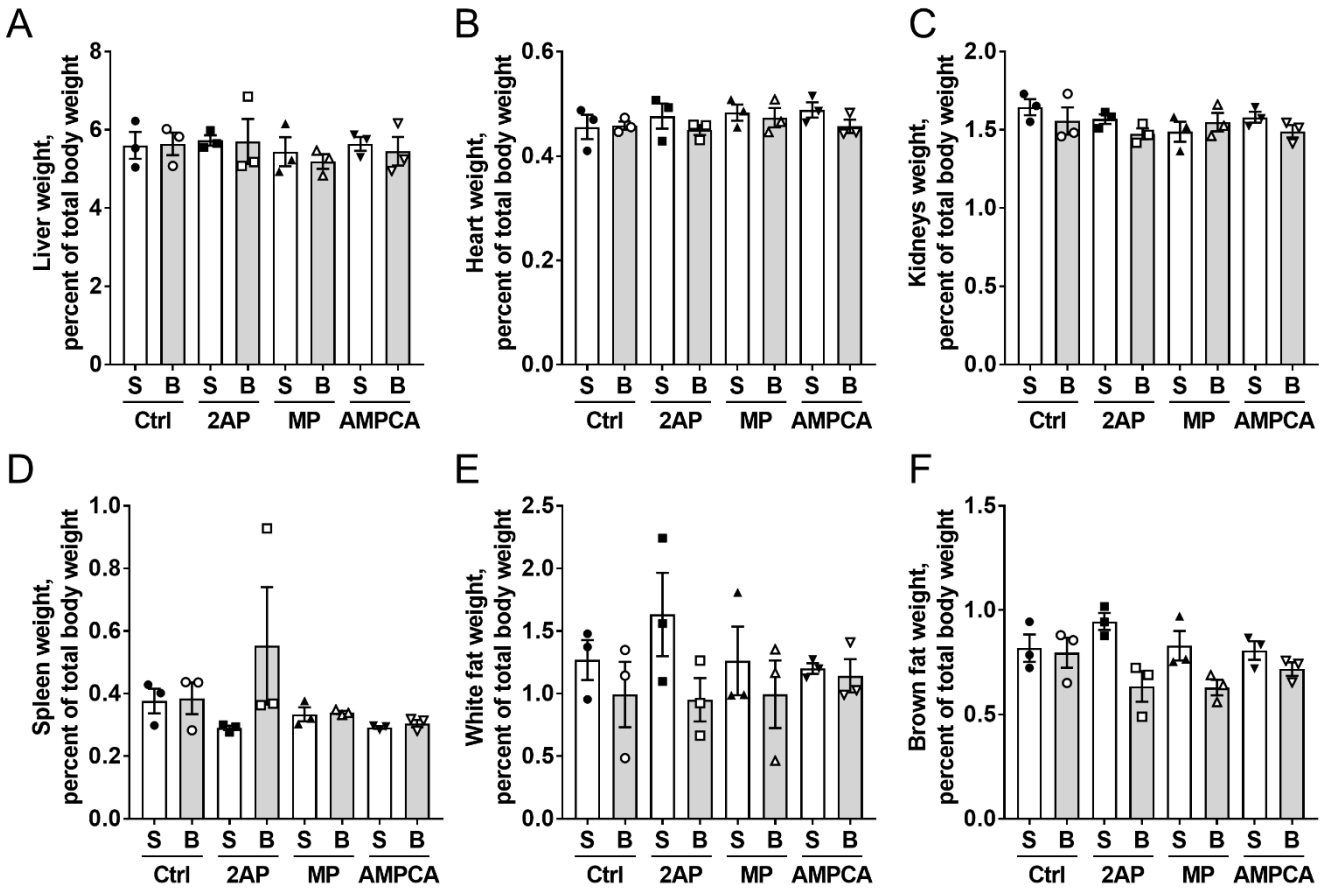
Supplementary Figure 5. The effect of sialidases on extracellular accumulation of IL-6. Human peripheral blood mononuclear cells (PBMCs) were cultured with the indicated recombinant human sialidases for 24 hours and 48 hours, and culture supernatants were assayed by ELISA for IL-6. Values are mean ± SEM, n = 3. * $p < 0.05$ (t-test).



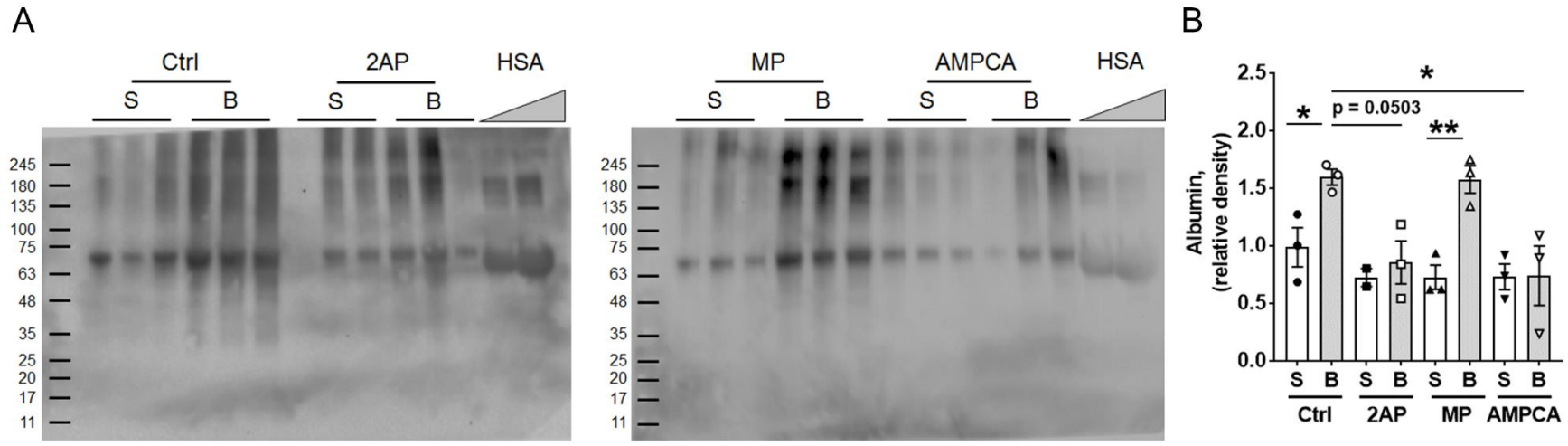
Supplementary Figure 6. Inhibition by compounds from Table 1 of recombinant human NEU3-induced extracellular accumulation of IL-6 from human PBMCs. Compounds labelled on the X-axis of graphs **(A)** to **(E)** were incubated at the indicated concentrations with and without recombinant human NEU3 in serum free media. After 30 minutes, human PBMCs were added and incubated at 37 °C, 5% CO_2. 48 hours later media supernatants were assayed for IL-6. Values are mean ± SEM, n ≥ 3. The NEU3-induced extracellular accumulation of IL-6 was determined by subtracting IL-6 values in the absence of NEU3 from IL-6 values in the presence of NEU3 in the data from (A – E). The baseline corrected values were normalized to the percent of IL-6 value in the absence of the compound (0 μM of inhibitor). **(F) – (J)**. IC_{50} values (red lines) were determined from the normalized values by non-linear regression curve fitting. Values are mean ± SEM, n ≥ 3.



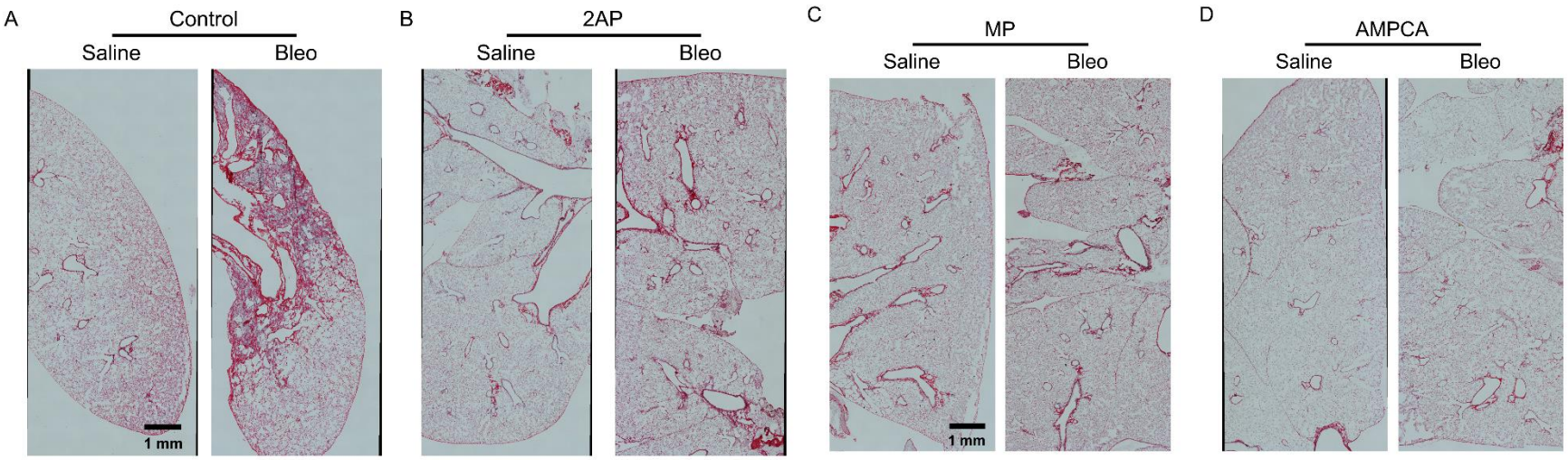
Supplementary Figure 7. NEU3 inhibitors inhibit recombinant mouse NEU3-catalyzed release of active TGF-β1. (A-C) The indicated compounds were incubated with and without recombinant mouse NEU3. rhL-TGF-β1 was added as a substrate, and the release of active TGF-β1 was quantified. Values are mean ± SEM, n ≥ 3. **(D-F)** IC_{50} values were determined as described for Supplementary Fig. 4. Values are mean ± SEM, n ≥ 3.



Supplementary Figure 8. Injections of 2AP, MP, or AMPCA from day 10 after bleomycin treatment had no significant effect on organ weights. Weights of **(A)** Liver, **(B)** Heart, **(C)** Kidneys, **(D)** Spleen, **(E)** White fat, and **(F)** Brown fat as percent of total body weight at day 21 after saline (S) or bleomycin (B) treatment. Values are mean ± SEM, n = 3. There were no significant changes (1way ANOVA, Bonferroni's test).



Supplementary Figure 9. AMPCA attenuates bleomycin-induced upregulation of albumin in the BAL fluid at day 21 after bleomycin treatment. (A) BAL fluids from the indicated mice were analyzed by western blots stained with anti-albumin antibodies. Molecular mass markers in kDa are at left and in the center. Human serum albumin (HSA) at 1 and 10 μg was loaded at right on each gel. **(B)** Quantification of protein by densitometry in lanes from panel A, using the BSA band densities as standards, and then for each mouse multiplying by the BAL volume to obtain relative albumin levels. S indicates saline and B indicates bleomycin. Values are mean ± SEM, n=3 except for saline aspirated and 2-acetyl pyridine (2AP) treated mice where n=2. * $p < 0.05$ and ** $p < 0.01$ (t-test).



Supplementary Figure 10 Picrosirius red staining of lung cryosections. Representative mosaic images of mouse lung cryosections stained with Sirius red. Images are representative of 3 mice aspirated with saline or bleomycin and injected with (**A**) PBS as control, (**B**) 2-acetyl pyridine (2AP), (**C**) Methyl picolinate (MP), or (**D**) 4-amino-1-methyl-2-piperidine carboxylic acid (AMPCA).