Supporting Information

Alleviation of Multiple Asthmatic Pathologic Features with Orally Available and Subtype Selective $GABA_A$ Receptor Modulators

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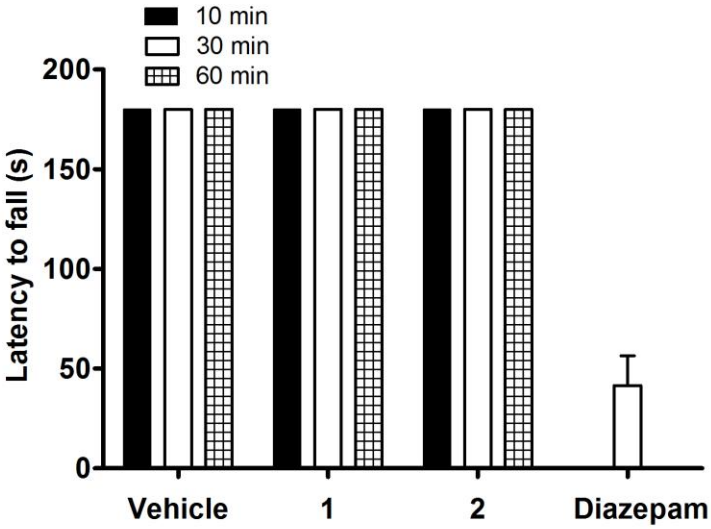

Figure S1 (related to Figure 5). Effect of 1 and 2 on sensorimotor coordination. Swiss Webster mice received a single intra-gastric gavage of test compound (100 mg/kg) or diazepam (5 mg/kg ip) and placed on a rotarod at 15rpm for 3 minutes after 10, 30 and 60 minutes of drug administration. A fail was assigned to a mouse having fallen twice prior to 3 minutes. The latency to fall is expressed as mean ± SEM from 9 mice in each group. Vehicle and diazepam was used as negative and positive control respectively.

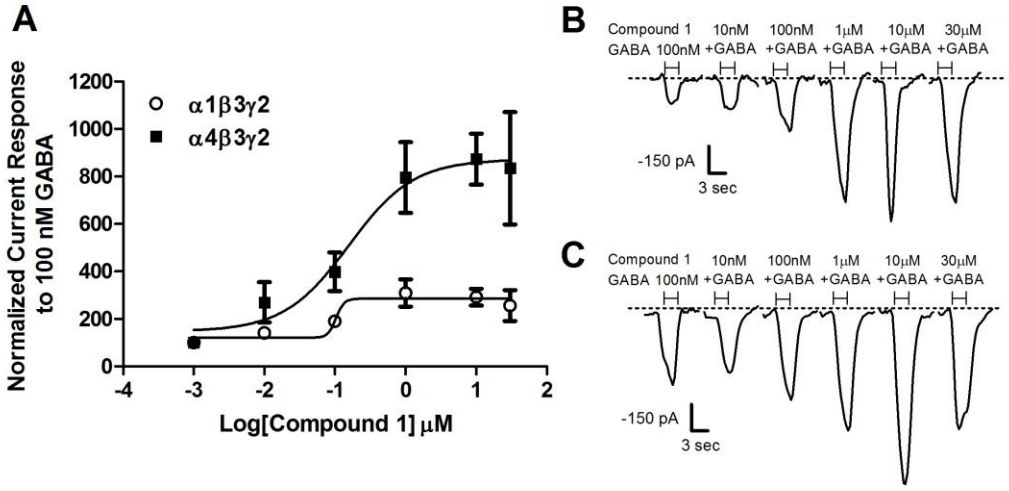

Figure S2 (related to Figure 5). Automated patch clamp with compound 1. A) Concentration-dependent negative current responses in the presence of EC_{20} concentration of GABA and increasing concentration of compound 1 applied together for 3 seconds using α1β3γ2 or α4β3γ2 $GABA_AR$ expressing HEK293T cells. Negative current readings were normalized to EC_{20} concentration of GABA response set as 100% (n = 16). B) Current recordings in the presence of EC_{20} concentration of GABA and increasing concentrations of compound 1 applied together for 3 seconds using α4β3γ2 expressing HEK293T cells. C) Current recordings in the presence of EC_{20} concentration of GABA and increasing concentrations of compound 1 applied together for 3 seconds using α1β3γ2 expressing HEK293T cells.

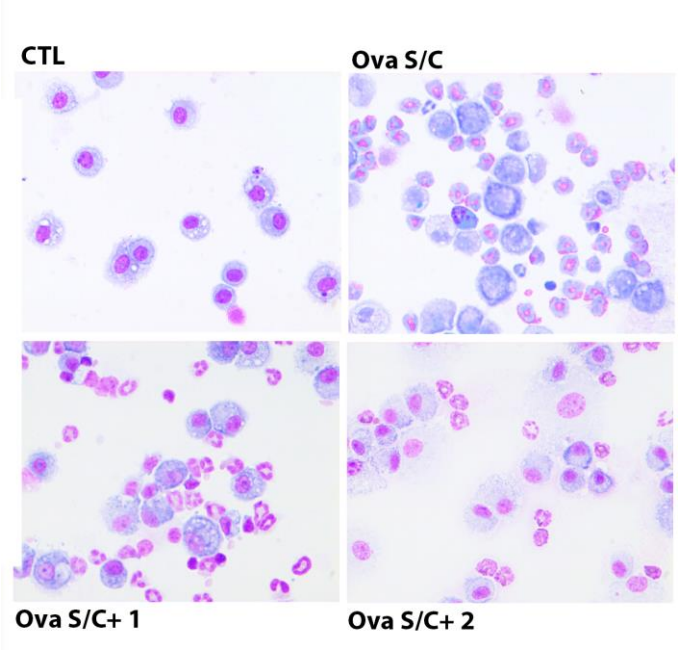

Figure S3 (related to Figure 6B, where quantification is displayed).
Effect of 1 and 2 on airway eosinophilia in BALF.
Representative images of Wright Giemsa stained slides. Ova s/c BALB/c mice were administered 1 or 2 via oral gavage, 100 mg/kg twice daily for 5 days. Data represent % normalized eosinophils relative to CTL (negative control) and ova s/c mice (positive control) from 11 mice in each group. ∗ and ∗∗∗ indicate $p < 0.05$ and $p < 0.001$ significance, respectively compared to vehicle treated ova s/c mice.

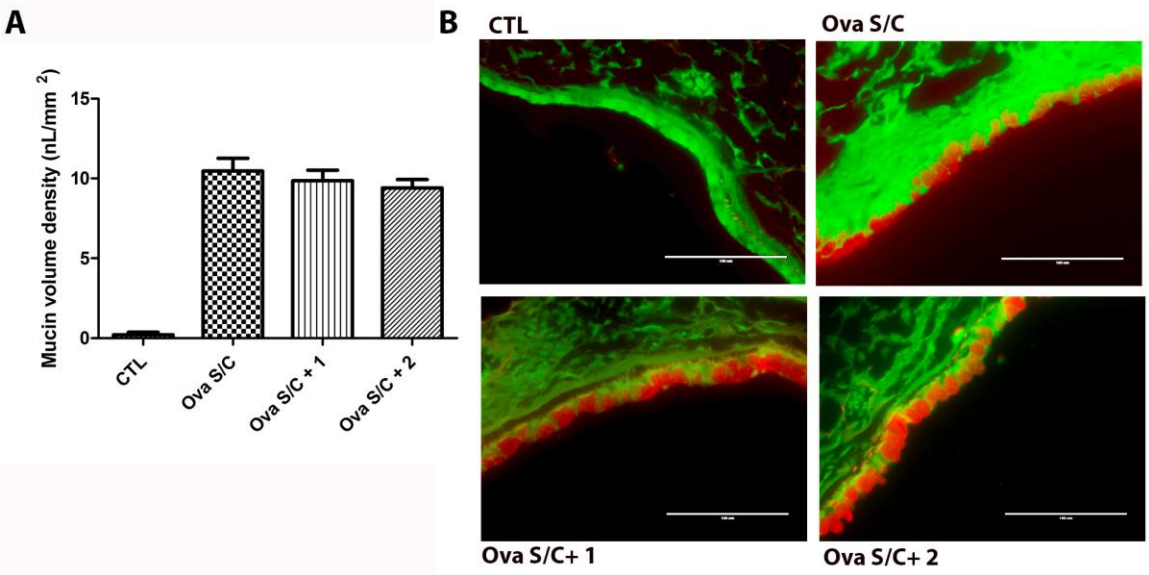

Figure S4 (related to Figure 6). Effect of 1 and 2 on mucous production
Morphometric quantification of mucin volume density and B) representative images of mucin (red) in the airway epithelium (green) with periodic acid fluorescent Schiff's stain. Ova s/c BALB/c mice were administered 1 via oral gavage, 100 mg/kg twice daily for 5 days or 2 via oral gavage, 100 mg/kg twice daily for 5 days. Data represent mean ± SEM mucin volume density from 6 mice in each group. Scale bar represents 100 μm.

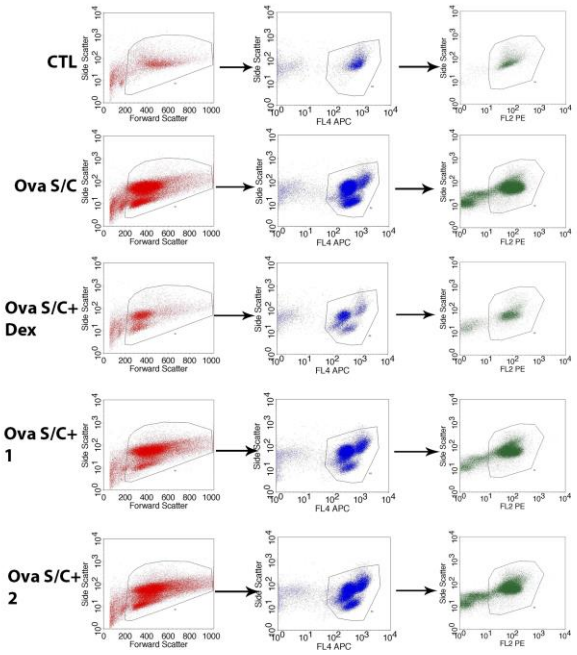

Figure S5 (related to Figure 6). Gating strategy for CCR3+ cells in BALF of ova s/c BALB/c mice administered dexamethasone i.p., 4 mg/kg daily for 8 days; 1 via oral gavage, 100 mg/kg twice daily for 5 days and 2 via oral gavage, 100 mg/kg twice daily for 5 days.

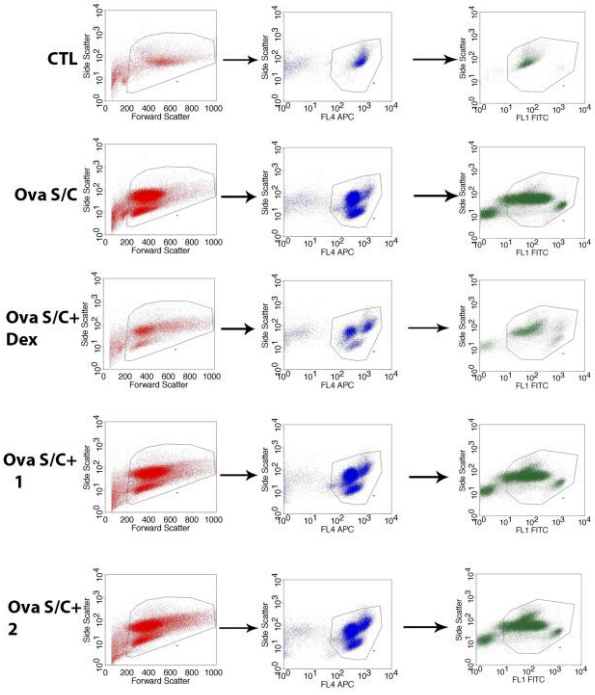

Figure S6 (related to Figure 6). Gating strategy for GR1+ cells in BALF of ova s/c BALB/c mice administered dexamethasone i.p., 4 mg/kg daily for 8 days; 1 via oral gavage, 100 mg/kg twice daily for 5 days and 2 via oral gavage, 100 mg/kg twice daily for 5 days.

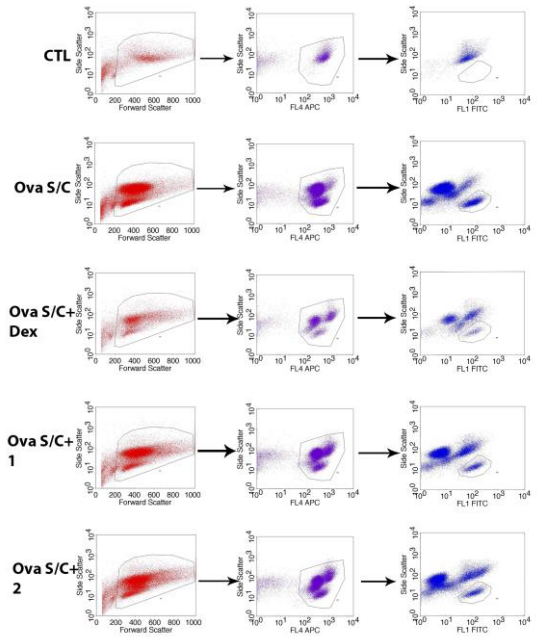

Figure S7 (related to Figure 6). Gating strategy for CD4+ cells in BALF of ova s/c BALB/c mice administered dexamethasone i.p., 4 mg/kg daily for 8 days; 1 via oral gavage, 100 mg/kg twice daily for 5 days and 2 via oral gavage, 100 mg/kg twice daily for 5 days.

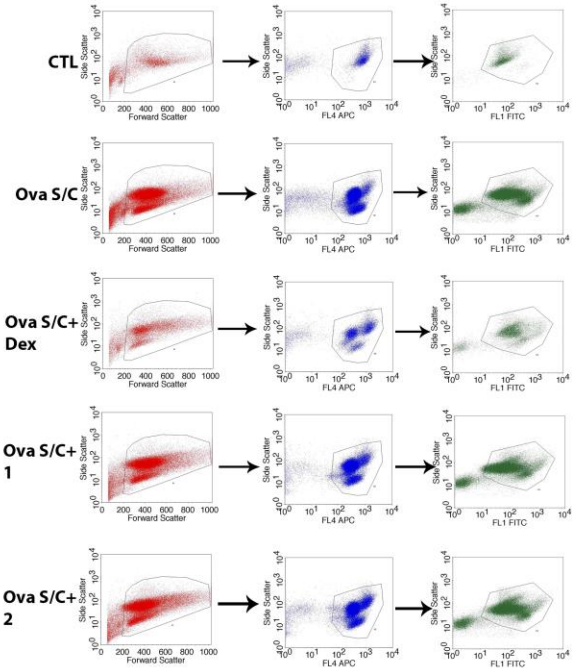

Figure S8 (related to Figure 6). Gating strategy for CD11b+ cells in BALF of ova s/c BALB/c mice administered dexamethasone i.p., 4 mg/kg daily for 8 days; 1 via oral gavage, 100 mg/kg twice daily for 5 days and 2 via oral gavage, 100 mg/kg twice daily for 5 days.

Characterization of compound 1.

(S)-Ethyl-7-hydroxy-9-oxo-11,12,13,13a-tetrahydro-9H-benzo[e]imidazo[5,1-c]pyrrolo[1,2-a][1,4]diazepine-1-carboxylate: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (t, 3H, *J* = 7.1 Hz), 2.19-2.42 (m, 3H), 3.55-3.64 (m, 2H), 3.81-3.89 (m, 1H), 4.42 (q, 2H, *J* = 7.1 Hz), 4.82 (d, 1H, *J* = 7.3 Hz), 7.13 (dd, 1H, J = 8.7 Hz, 2.6 Hz), 7.27-7.31 (m, 1H), 7.85 (s, 1H), 7.91 (d, 1H, J = 2.6 Hz), 9.22 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 14.3, 24.4, 28.4, 46.9, 53.8, 61.2, 117.5, 120.8, 124.9, 125.2, 127.7, 129.5, 136.0, 137.2, 157.6, 162.8, 164.6; HRMS (ESI) $(M+H)^+$, calcd. for $C_{17}H_{18}N_3O_4$ 328.1292; Found 328.1293.

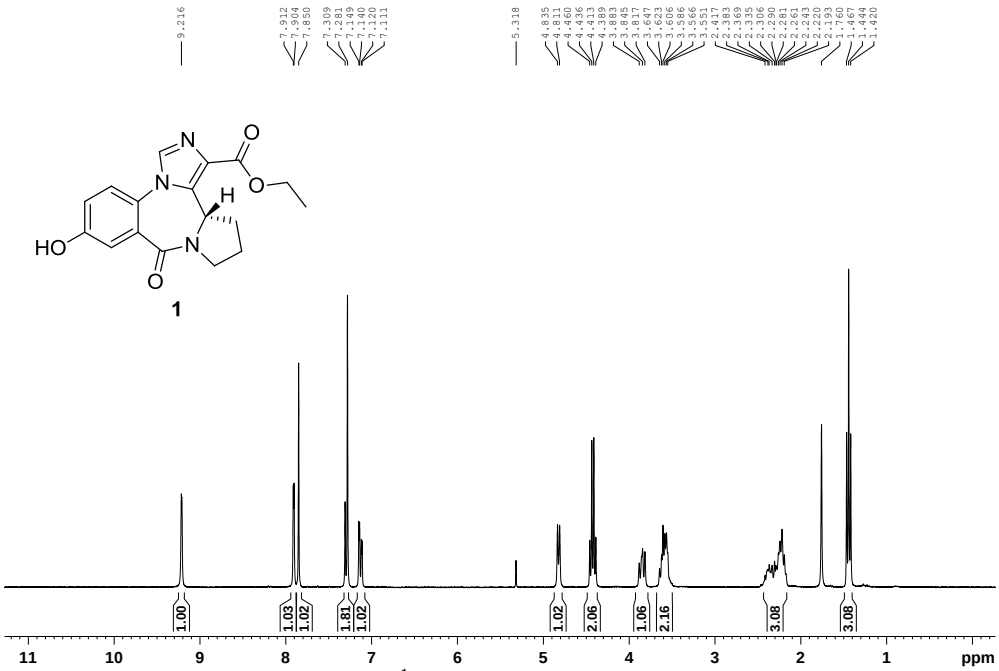

Figure S9 (related to Figure 1). ^{1}H-NMR spectra of 1.

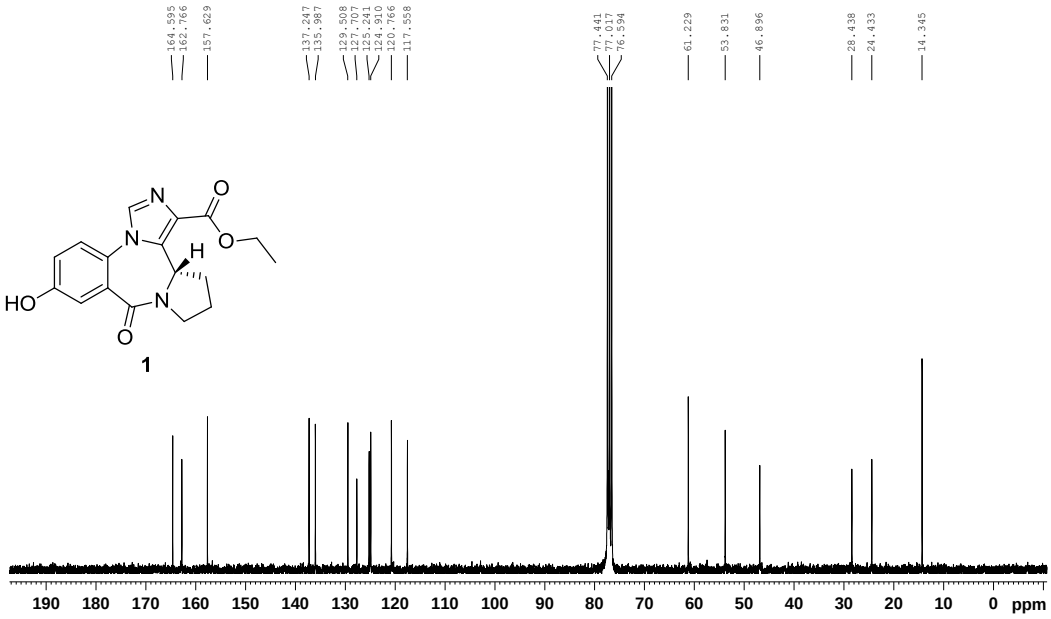

Figure S10 (related to Figure 1). ^{13}C-NMR spectra of 1.

100% Purity (5-95% acetonitrile in water)

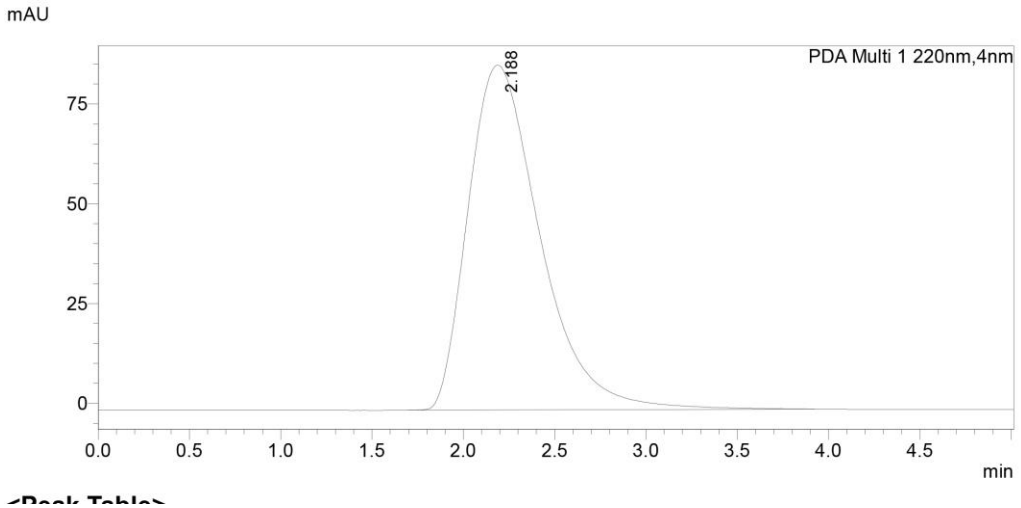

Figure S11 (related to Figure 1). HLPC run of 1.

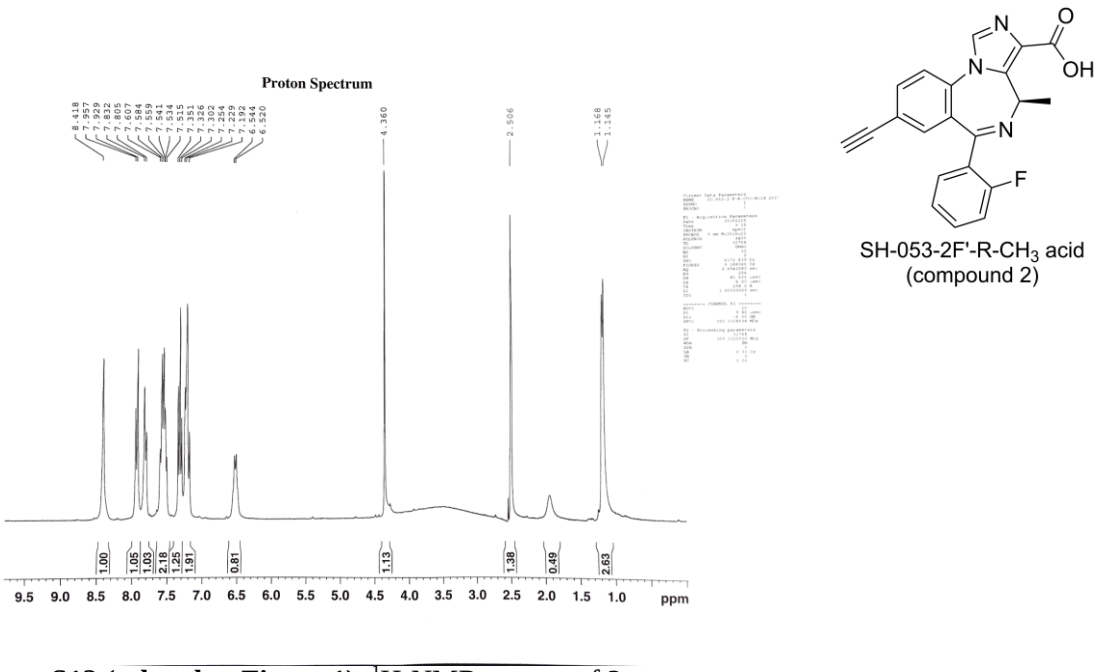

Figure S12 (related to Figure 1). ^{1}H-NMR spectra of 2.

Characterization of compound 2

(*R*)-8-ethynyl-6-(2-fluorophenyl)-4-methyl-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylic acid.

^{1}H NMR (300 MHz, DMSO-d^6) δ 8.42 (s, 1H), 7.94 (d, 1H, J = 8.4 Hz), 7.82 (d, 1H, J = 8.2 Hz), 7.56 (dt, 2H, J = 7.8, 6.5 Hz), 7.33 (t, 1H, J = 7.4 Hz), 7.22 (t, 2H, J = 9.3 Hz), 6.53 (d, 1H, J = 7.1 Hz), 2.51 (s, 1H), 1.16 (d, 3H, J = 6.8 Hz); ^{13}C NMR (75 MHz, DMSO-d^6) δ 164.76, 162.81, 158.19, 140.57, 136.57, 135.54, 134.74, 133.18, 132.65, 131.88, 129.88, 129.35, 125.17, 123.98, 121.09, 116.53, 116.25, 83.42, 82.01, 49.79, 15.08; HRMS (LCMS-IT-TOF) Calc. for $C_{21}H_{14}FN_3O_2$ $(M + H)^+$ 360.1143, found 360.1140.

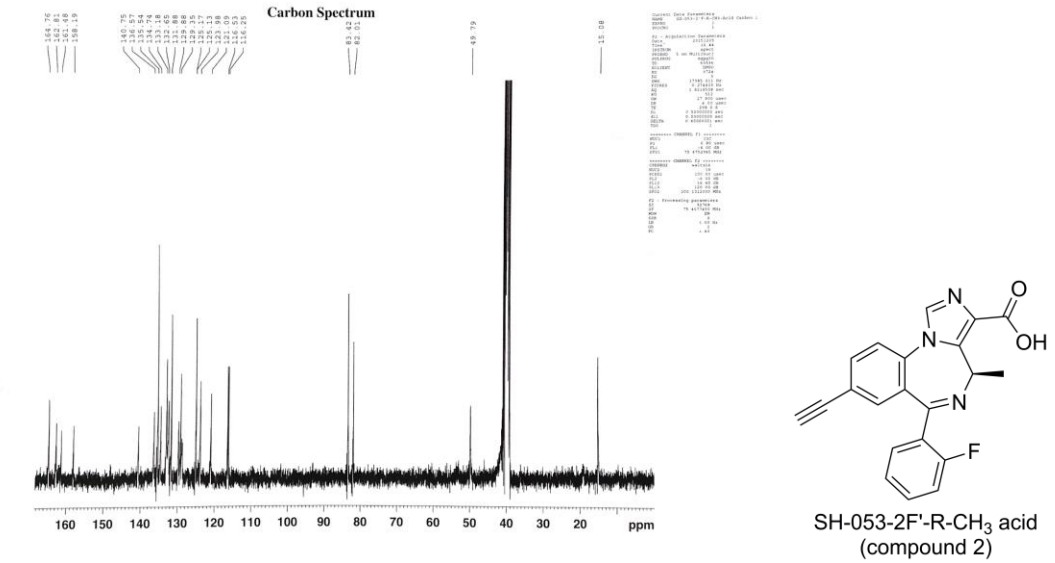

Figure S13 (related to Figure 1). ^{13}C-NMR spectra of 2.

100% Purity (50-95% acetonitrile in water)

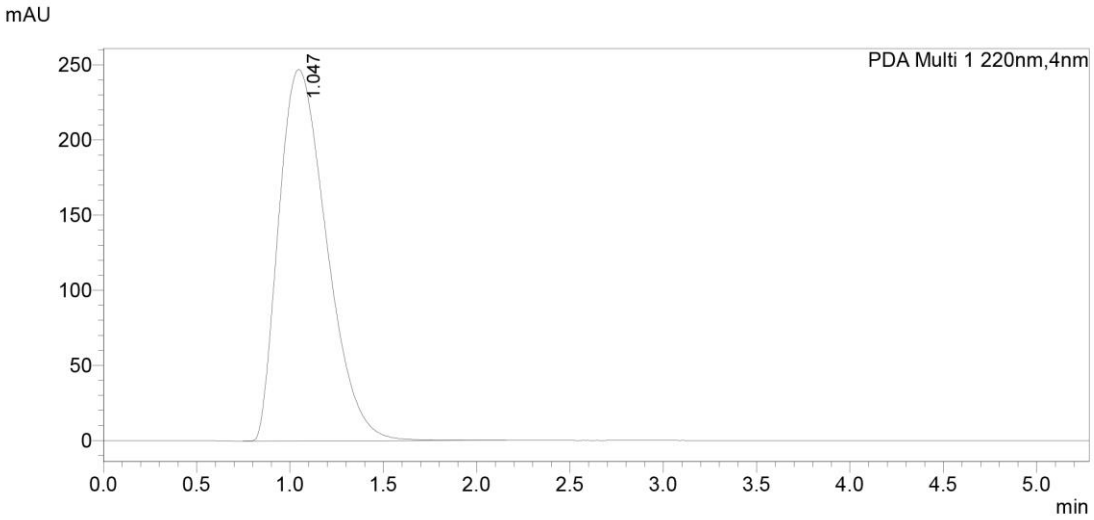

Figure S14 (related to Figure 1). HPLC run of 2.

Characterization of compound 2

(*R*)-8-ethynyl-6-(2-fluorophenyl)-4-methyl-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carboxylic acid.

^{1}H NMR (300 MHz, DMSO-d^6) δ 8.42 (s, 1H), 7.94 (d, 1H, J = 8.4 Hz), 7.82 (d, 1H, J = 8.2 Hz), 7.56 (dt, 2H, J = 7.8, 6.5 Hz), 7.33 (t, 1H, J = 7.4 Hz), 7.22 (t, 2H, J = 9.3 Hz), 6.53 (d, 1H, J = 7.1 Hz), 2.51 (s, 1H), 1.16 (d, 3H, J = 6.8 Hz); ^{13}C NMR (75 MHz, DMSO-d^6) δ 164.76, 162.81, 158.19, 140.57, 136.57, 135.54, 134.74, 133.18, 132.65, 131.88, 129.88, 129.35, 125.17, 123.98, 121.09, 116.53, 116.25, 83.42, 82.01, 49.79, 15.08; HRMS (LCMS-IT-TOF) Calc. for $C_{21}H_{14}FN_3O_2$ $(M + H)^+$ 360.1143, found 360.1140.

Supplemental experimental procedures

Airway Smooth Muscle relaxation. All studies were conducted after approval of the Columbia University IACUC. Adult male Hartley guinea pigs were euthanized by intraperitoneal pentobarbital (100 mg/kg). The tracheas were surgically removed and transected into cross-sections containing two cartilaginous rings. The rings are washed for one hour with at least five buffer exchanges to remove any pentobarbital. After the epithelium was removed with a cotton swab, the rings were suspended from two silk threads in a 4 mL jacketed organ bath (Radnoti Glass Technology), with one thread attached to a Grass FT03 force transducer (Grass-Telefactor) coupled to a computer via Biopac hardware and Acknowledge 7.3.3 software (Biopac Systems) for continuous digital recording of muscle tension. The rings were bathed in 4 ml of KH buffer solution (composition in mM: 118 NaCl, 5.6 KCl, 0.5 $CaCl_2$, 0.2 $MgSO_4$, 25 $NaHCO_3$, 1.3 NaH_2PO_4, 5.6 D-glucose) with 10 μM indomethacin (DMSO vehicle final concentration of 0.01%), which was continuously bubbled with 95% O_2 and 5% CO_2 at pH 7.4, 37 °C. The rings were equilibrated at 1 g of isotonic tension for 1 h with new KH buffer added every 15 min. All rings were precontracted with 10 μM N-vanillylnonanamide (capsaicin analog) and then two cycles of cumulatively increasing concentrations of acetylcholine (0.1−100 μM) with extensive buffer washes between and after those two cycles with resetting of the resting tension to 1.0 g. Tetrodotoxin (1 μM) and pyrilamine (10 μM) were added to the buffer in the baths to eliminate the confounding effects of airway nerves and histamine receptors. After a stable baseline at 1.0 g resting tension was established, tracheal rings were contracted with 1 μM of substance P. After the peak contraction was reached, indicated concentrations of 1 or 2, or vehicle (0.1% DMSO) was added to the bath. The percentage of initial contraction remaining at indicated time points after compound exposure was expressed as a percentage of the remaining contractile force in vehicle-treated tissues and compared between groups.

Human airway smooth muscle strips were dissected from human trachea obtained from healthy donor lungs incidental to lung transplantations. Studies were reviewed by the Columbia University IRB and deemed not to be human subject research. Strips were suspended as above in organ baths in oxygenated KH buffer at 37 °C at 1.5g of resting tension. Following equilibration for 1h with buffer exchanges every 15 min, strips were contracted with 3 cycles of increasing concentrations of acetylcholine (100 nM - 1 mM) will extensive buffer exchanges between and after these pre-contractile challenges. MK571 (10 μM), pyrilamine (10 μM) and tetrodotoxin (1 μM) were added to the buffer before each strip was contracted to its individually calculated EC_{50} concentration of acetylcholine. When a plateau in the increase in contractile force was achieved (typically 15 min) 100 μM of 2 or its vehicle (0.2% ethanol) was added to the buffer and the maintenance of contractile force was continuously measured over 1h. The remaining contractile force at 15, 30, 45 and 60 min was expressed as a percentage of the initial acetylcholine-induced contractile force.

Rotarod assay: Swiss Webster mice were trained to maintain balance at a constant speed of 15 rpm on the rotarod apparatus (Omnitech Electronics Inc., Nova Scotia, Canada) until mice could

perform for 3 minutes at three consecutive time points. Separate groups of nine mice received oral gavage vehicle (2% hydroxypropyl methylcellulose and 2.5% polyethylene glycol) or test compounds (1 and 2, 100 mg/kg) in an approximate volume of 200 µl. Control compound diazepam was given as an ip injection at 5 mg/kg in 10% DMSO, 40% propylene glycol, and 50% PBS. The mice were placed on the rotarod at three separate time points of 10, 30, and 60 minutes after each oral gavage drug administration. A fail was classified for each mouse falling twice prior to 3 minutes since it was common for a mouse injected with vehicle to occasionally fall once. Hence after a second fall, it would be considered a fail, and that time point would be recorded. Mice were rested two to three days before administration of another dose or a different compound.

Patch clamp assay: HEK293T stably expressing α1β3γ2 $GABA_AR$ or α4β3γ2 were maintained RPMI 1640 medium with L-glutamine supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin. Automated patch-clamp studies were conducted as described previously.(Forkuo et al., 2016) Briefly, the IonFlux plate layout consists of units of 12 wells: two wells contain intracellular solution (ICS containing 140 mM CsCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 11 mM EGTA, 10 mM HEPES, pH 7.2 with CsOH), one contains cells diluted in extracellular solution (ECS containing 140 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 10 mM D-glucose monohydrate, and 10 mM HEPES, pH 7.4 with NaOH), eight contain different concentration of 1 in the presence of GABA (EC_{20} concentration) at 0.1% DMSO. Well 1 is for waste collection. Cells are captured from suspension by applying suction to microscopic channels in ensemble recording arrays. Once the array is fully occupied, the applied suction breaks the membranes of captured cells, which establishes whole cell voltage clamp. For compound applications, pressure is applied to the appropriate compound wells, which introduces the compound into the extracellular solution rapidly flowing over the cells. For recording $GABA_AR$ induced currents, cell arrays were voltage clamped at a hyperpolarizing holding potential of −80 mV. Prior to use on the automated patch clamp, cells were centrifuged at 380g for 5 minutes and resuspended gently in ECS. This was repeated two more times before the cells were dispensed into the plate. All compound application were carried out for 3 seconds followed by a 5 second washout.

Histopathological analysis of lung sections: After BALF collection, the lungs were perfused with 10% neutral buffered cold formalin (Sigma-Aldrich, St. Louis, MO) through the tracheal cannula. Following lung perfusion, the trachea was tied with a suture to avoid leakage of the formalin and to ensure the lungs are well fixed. The lungs were then isolated from the thoracic cavity and kept in 10% neutral buffered cold formalin for 48 hours at 4^0C. The left lobe was then sectioned transversely into two. Lung samples were sent over to CRI Histology Research Core Facility (Milwaukee, WI) for dehydration, paraffin embedding and sectioning. 5 µm sections of paraffin embedded lungs were prepared unto positively charged slides using the microtome. The sections were dewaxed with histoclear and rehydrated in graded concentrations of ethanol. Following rehydration, the sections were oxidized in 1% periodic acid and incubated in fluorescent Schiff's reagent for 20minutes at room temperature. The slides were washed with distilled water, rinsed in acid alcohol and cover slipped with canada balsam and methyl salicylate mounting media to obtain Periodic Acid Fluorescent Schiff's (PAFS) stained slides (Evans et al., 2004; Piccotti et al., 2012). The PAFS-stained slides were examined under the EVOS fluorescence microscope and images from random fields of the lung sections were acquired from the axial bronchi. The image J software was used to obtain mucin volume density by morphometrically determining the area of mucin glycoprotein in the epithelium per length of the basement membrane (Evans et al., 2004; Piccotti et al., 2012). Scale bars obtained during image acquisition were used to scale the images.

Mice Pharmacokinetic study: Female Swiss Webster mice received intra-gastric gavage of vehicle or either 1 or 2 formulated in 2% hydroxypropyl methylcellulose solution and 2.5% polyethylene glycol at a dose of 25 mg/kg. At each of the following time points 10, 20, 40 60,120,

240 and 480 minutes, four mice were sacrificed by carbon dioxide anesthesia followed by cervical dislocation. Blood (collected into heparinized tubes), lungs and brain were harvested and samples stored in liquid nitrogen until analysis.

Sample preparation and LC/MS: Blood samples were thawed on ice, vortexed for 10 seconds, and a 100 µl aliquot was taken and added to 400 µl cold acetonitrile containing [100 nM 4,5-diphenyl imidazole (for 1) and 100 nM HZ-166 (for 2)] internal standard (I.S.) Samples were vortexed for 30 seconds and centrifuged at 12,000 x g for 10 minutes. The supernatant layer was then transferred to clean tubes and evaporated using Speedvac concentrator. The residue was reconstituted with 400 µl of mobile phase and spin-filtered through 0.22 µm nylon centrifugal filter units (Costar). After reconstitution, the samples are properly diluted, the verapamil was added and 5 µl of the sample was injected to the LC–MS/MS. Brain and lung tissue samples were thawed, weighed, and homogenized directly into 400 µl ACN containing I.S. using a Cole Palmer LabGen 7B Homogenizer. Samples were centrifuged for 10 minutes at 12,000 x g. The supernatant was then retrieved, and prepared in the same manner as the blood samples for LC-MS/MS analysis.
High performance liquid chromatography (HPLC) was performed with Shimadzu Nexera X2 LC30AD series pumps (Shimadzu, Kyoto, Japan). Analytes were separated by a Restek Pinnacle II C18 column (2.1 mm × 100 mm, 5 um particle size, Restek, California, US) under gradient elution at a flow rate of 0.5 ml/min (1) and 0.4 ml/min (2). The mobile phase was acetonitrile and water (both containing 0.1% formic acid). Time program: 20% B → 70% B (3 min) →99% B (5 min), hold at 99% B (8 min), return to 10% B (9 min), hold (9.5 min) (1) and 70% B → 70% B (6 min), (Isocratic for 2) and column temperature: 40 °C.
Analytes were monitored under positive mode by Shimadzu 8040 triple quadrupole mass analyzer (Shimadzu, Kyoto, Japan) electrospray and atmospheric pressure ionization run in dual (DUIS) mode. The following transitions are monitored in multiple reaction monitoring (MRM) mode. Ion pairs for 1 are m/z 327.85 > 281.95, m/z 327.85 >264.05, m/z 327.85 > 254.10, m/z 327.85 > 236.80 and m/z 327.85 > 212.75. Transition ion pairs for 2 are m/z 360.0 > 342.10, m/z 360.0 > 316.00, m/z 360.0 > 301.10, m/z 360.0 > 249.05 and m/z 360.0 > 219.90. Transition pairs for HZ-166 are m/z 356.90 > 311.15, m/z 356.90 > 283.15, and m/z 356.90 > 282.15. Transition pairs for 4,5-diphenyl imidazole are m/z 220.80 > 193, m/z 220.80 > 167, m/z 220.80 >151.95 and m/z 220.80 > 115 transition pairs for verapamil (internal standard) are m/z 454.70 > 165.05, m/z 454.70 > 150 and m/z 454.70 > 303.0. Collision energy is optimized for each transition to obtain optimal sensitivity. The mass spectrometer was operated with the heat block temperature of 400°C, drying gas flow of 15 L/min, desolvation line temperature of 250°C, nebulizing gas flow of 1.5 L/min, and both needle and interface voltages of 4.5 kV. The response acquisition was performed using LabSolutions software. Standard curves were fitted by a linear regression and the validation samples were calculated back by the calibration curve of that day. The mean and the coefficient of variance (CV) were calculated accordingly. Accuracy was calculated by comparing calculated concentrations to corresponding nominal. Pharmacokinetic parameters were calculated with PK solutions software 2.0 and fitted to the following equation: $c = A \bullet e^{-at} + B \bullet e^{-bt} + C \bullet e^{-ct}$.

Reference

Evans, C.M., Williams, O.W., Tuvim, M.J., Nigam, R., Mixides, G.P., Blackburn, M.R., DeMayo, F.J., Burns, A.R., Smith, C., Reynolds, S.D., *et al.* (2004). Mucin is produced by clara cells in the proximal airways of antigen-challenged mice. Am J Respir Cell Mol Biol *31*, 382-394.
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Piccotti, L., Dickey, B.F., and Evans, C.M. (2012). Assessment of intracellular mucin content in vivo. Methods Mol Biol *842*, 279-295.