Supporting Information

A novel orally available asthma drug candidate that reduces smooth muscle constriction and inflammation by targeting $GABA_A$ receptors in the lung

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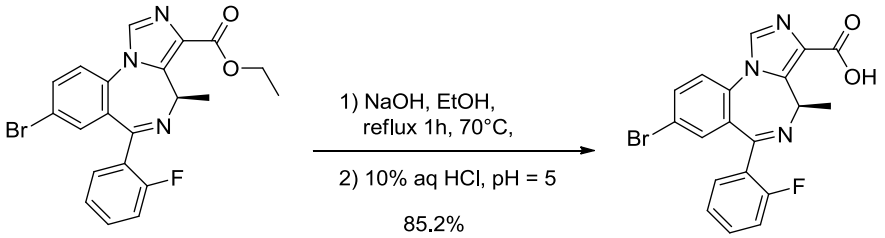
Table S1. Summary of microsomal stability studies for MIDD0301

MIDD0301	Human Liver Microsomes	Mouse Liver Microsomes
Half-life (min)	1546 ± 501	549 ± 81
V_d (µL/mg)	2000	2000
Internal Clearance (µL/min/mg)	0.0463	0.1312
Metabolic Rate (nmol/min/mg)	0.9261	2.624
% remaining at end of 2 hrs. (%)	91 ± 0.25	83 ± 0.20

Table S2. Summary of mouse plasma protein binding data

Result %		
MIDD0301	Unbound drug	Bound drug
Average (n = 3)	11.75 ± 1.42	88.25 ± 1.42



SH-I-47 GL-II-93 (compound **4**)

Scheme S1. Synthesis of MIDD0301.

(R)-8-Bromo-6-(2-fluorophenyl)-4-methyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylic acid (MIDD0301). The ethyl ester SH-I-47[1] (5.1 g, 11.5 mmol) was dissolved in EtOH (150 mL), after which solid NaOH (0.9 g, 23 mmol) was added to the solution. This reaction mixture was heated to 70 ˚C for 1 h and the EtOH was removed under reduced pressure. The remaining aq solution was stirred at 0˚C for 10 min and then 10 % aq HCl was added dropwise to the solution until the pH was 5 (pH paper). The pale white precipitate that formed was left in the solution for 10 min and then collected by filtration. The solid was washed with cold water and the

aq layer also allowed to stand at rt for 10 h to yield additional acid. The combined solids were dried in a vacuum oven at 80˚C for 7 h to provide pure acid MIDD0301 as a white powder (4 g, 9.7 mmol, 85.2%): ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.12 (s, 1H), 7.73 (d, J = 7.6 Hz, 1H), 7.63 (t, J = 6.9 Hz, 1H), 7.55 (d, J = 8.3 Hz, 1H), 7.45 (dd, J = 13.1, 6.7 Hz, 1H), 7.41 (s, 1H), 7.25 (t, J = 7.3 Hz, 1H), 7.06 (t, J = 9.3 Hz, 1H), 6.78 (q, J = 6.8 Hz, 1H), 1.27 (d, J = 6.3 Hz, 3H); ^{13}C NMR (500 MHz, $CDCl_3$): δ 165.00 (s), 162.89 (s), 160.09 (d, J = 251.0 Hz), 141.25 (s), 135.04 (s), 134.89 (s), 133.59 (s), 133.08 (s), 132.54 (d, J = 6.7 Hz), 132.16 (d, J = 7.8 Hz), 131.34 (s), 131.11 (s), 128.25 (d, J = 11.2 Hz), 124.57 (s), 123.91 (s), 121.09 (s), 116.22 (d, J = 21.3 Hz), 49.83 (s), 14.91 (s); HRMS (LCMS-IT-TOF) Calc. for $C_{19}H_{13}N_3O_2FBr$ (M + H)+ 414.0248, found 414.0235.

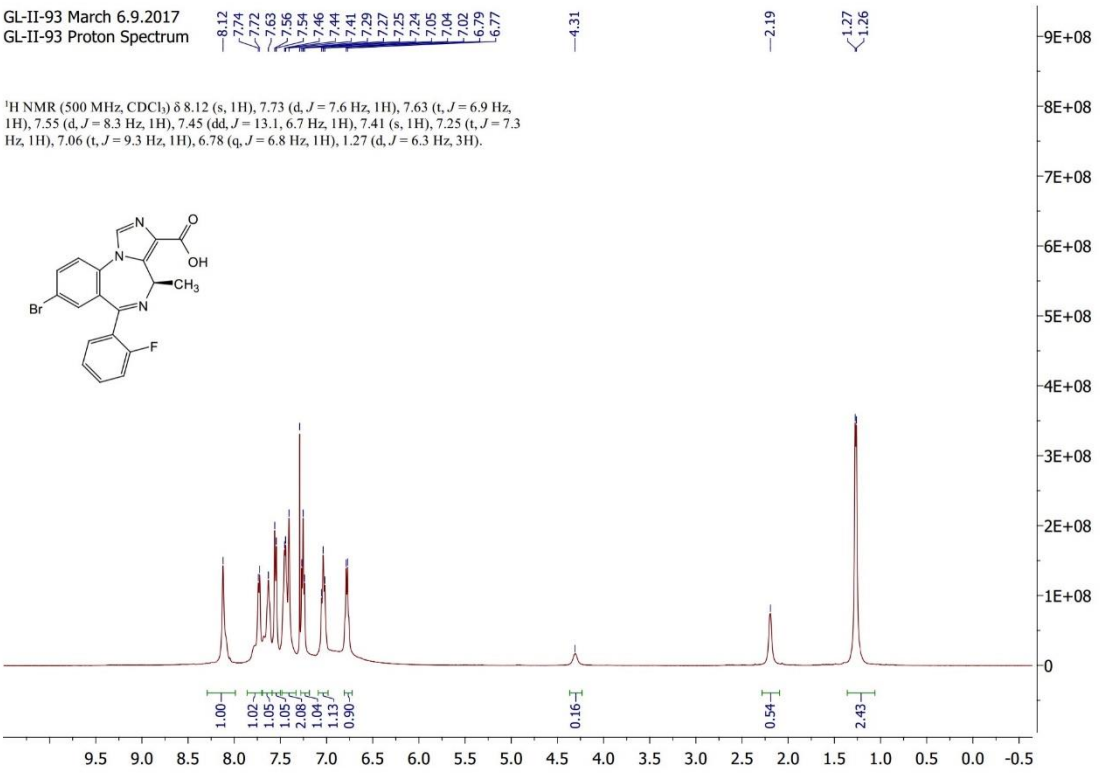


Figure S1. ^{1}H-NMR of MIDD0301

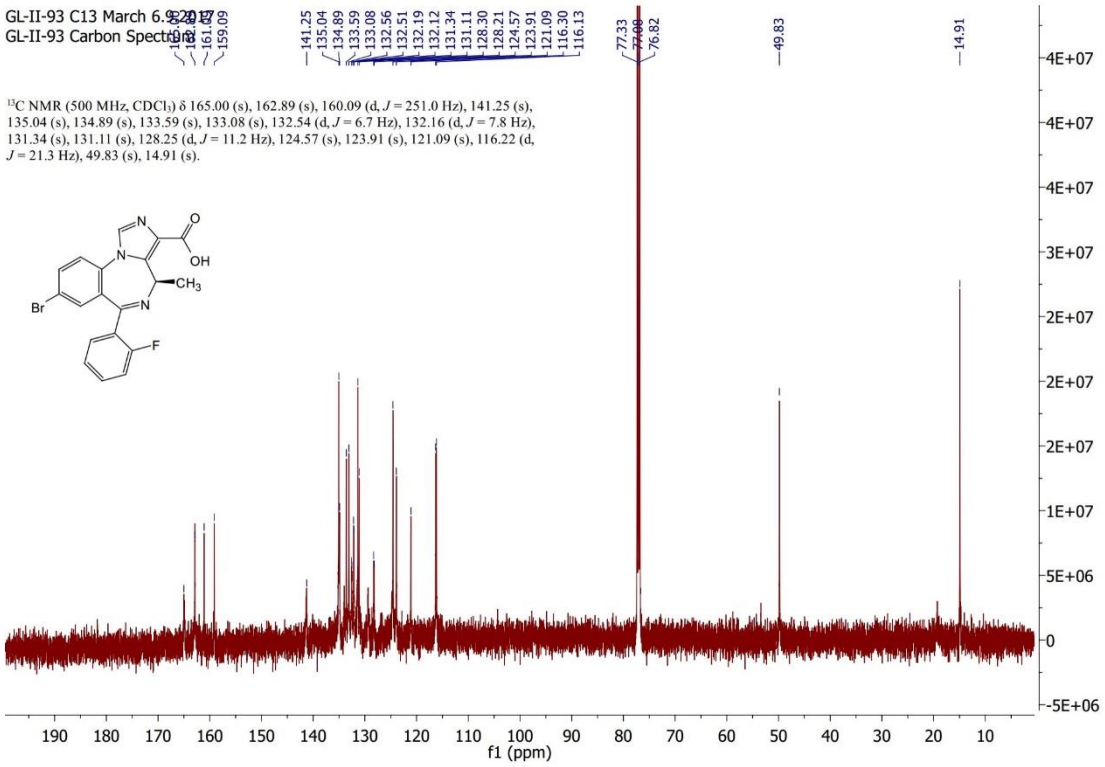
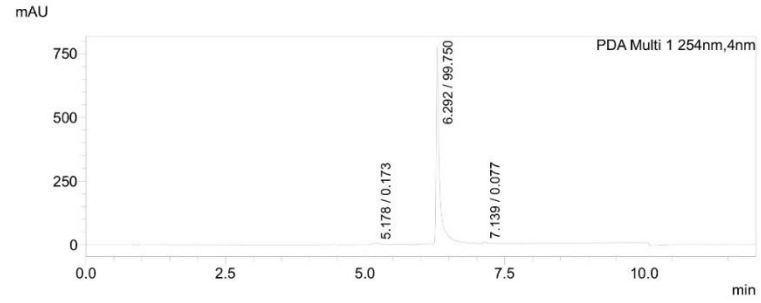


Figure S2. ^{13}C-NMR of MIDD0301

UWM Shimadzu LabSolutions HPLC.PDA Analysis Report

Sample Name	: GL-II-93		
Sample ID	: GL-II-93		
Data Filename	: GL-II-93_Gradient2 GL_7_001.lcd		
Method Filename	: Gradient2 GL.lcm		
Batch Filename	: 10-31-17.lcb		
Vial #	: 4-41	Sample Type	: Unknown
Injection Volume	: 2 uL		
Date Acquired	: 10/31/2017 4:43:28 PM	Acquired by	: Guanguan Li
Date Processed	: 11/2/2017 5:53:21 PM	Processed by	: Guanguan Li

<PDA Chromatogram>



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Pump A	: LC-30AD
Pump B	: LC-30AD
Total Flow	: 0.7000 mL/min
B Conc.	: 15.0 %
B Curve	: 0
PressMax	: 16000 psi
PressMin	: 0 psi
Solenoid Valve A Name	: FCV-15AL
Solenoid Valve A	: A
Solenoid Valve B Name	: FCV-15AL
Solenoid Valve B	: B
Compressibility Setting	: On
Pump A Compressibility	: 0.45 /GPa
Pump B Compressibility	: 1.25 /GPa

<<LC Time Program>>

Time	Module	Command
8.00	Pumps	Pump B Conc.
9.00	Pumps	Pump B Conc.
9.10	Pumps	Pump B Conc.
12.00	Controller	Stop

Peak Table

PDA Ch1 254nm

Peak#	Ret. Time	Area	Area%
1	5.178	5429	0.173
2	6.292	3128638	99.750
3	7.139	2409	0.077
Total		3136475	100.000

Figure S3. HPLC trace of MIDD0301

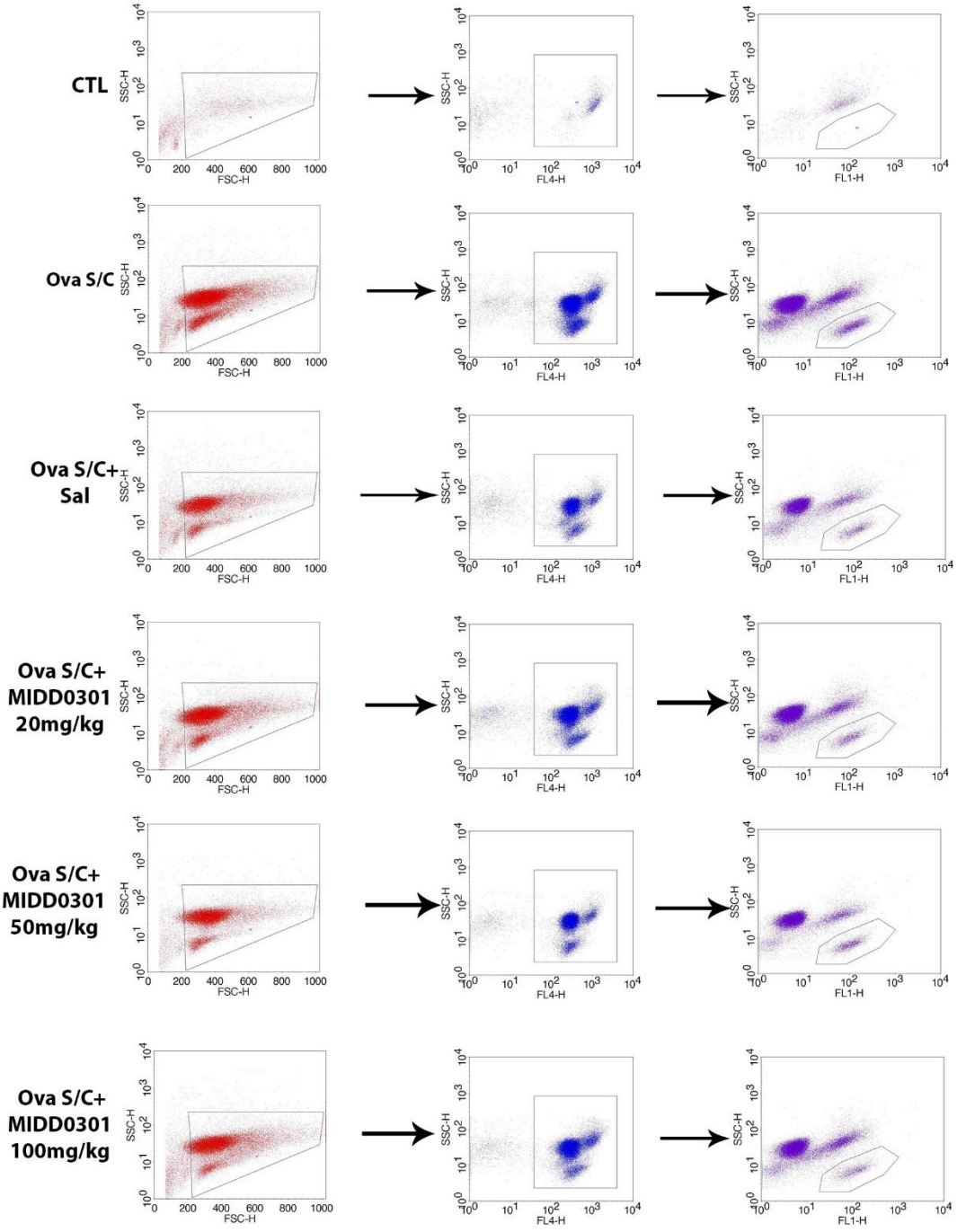

Figure S4. Gating strategy for CD4+ cells in BALF of Ova s/c mice administered vehicle or 100 mg/kg, 50 mg/kg, 20 mg/kg of MIDD0301 via oral gavage b.i.d. for 5 days or salmeterol also via oral gavage at 1 mg/kg b.i.d. for 5 days. (FL4-H = CD45 and FL1-H = CD4)

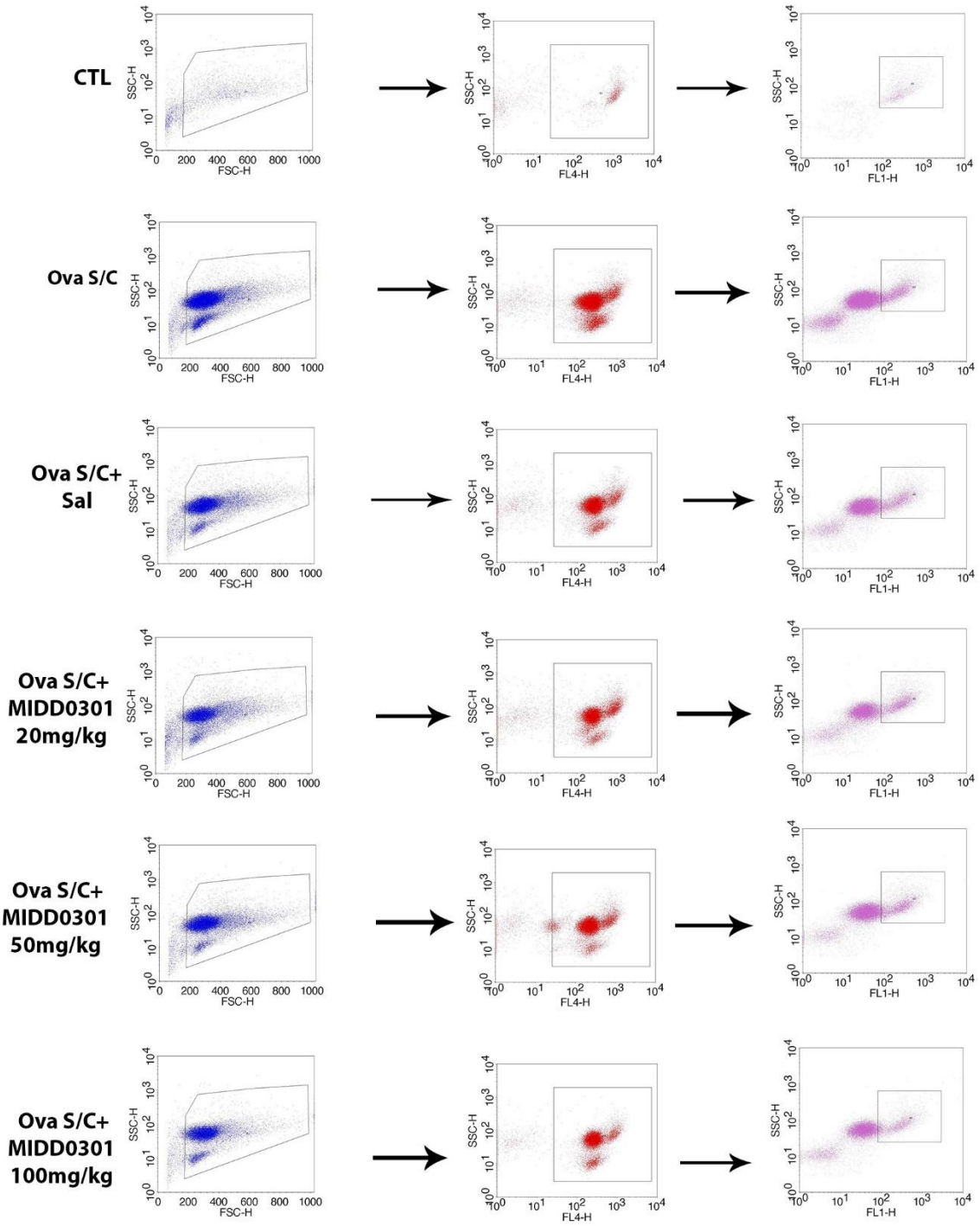

Figure S5. Gating strategy for F4/80+ cells in BALF of Ova s/c mice administered vehicle or 100 mg/kg, 50 mg/kg, 20 mg/kg of MIDD0301 via oral gavage b.i.d. for 5 days or salmeterol also via oral gavage at 1 mg/kg b.i.d. for 5 days. (FL4-H = CD45 and FL1-H = F4/80)

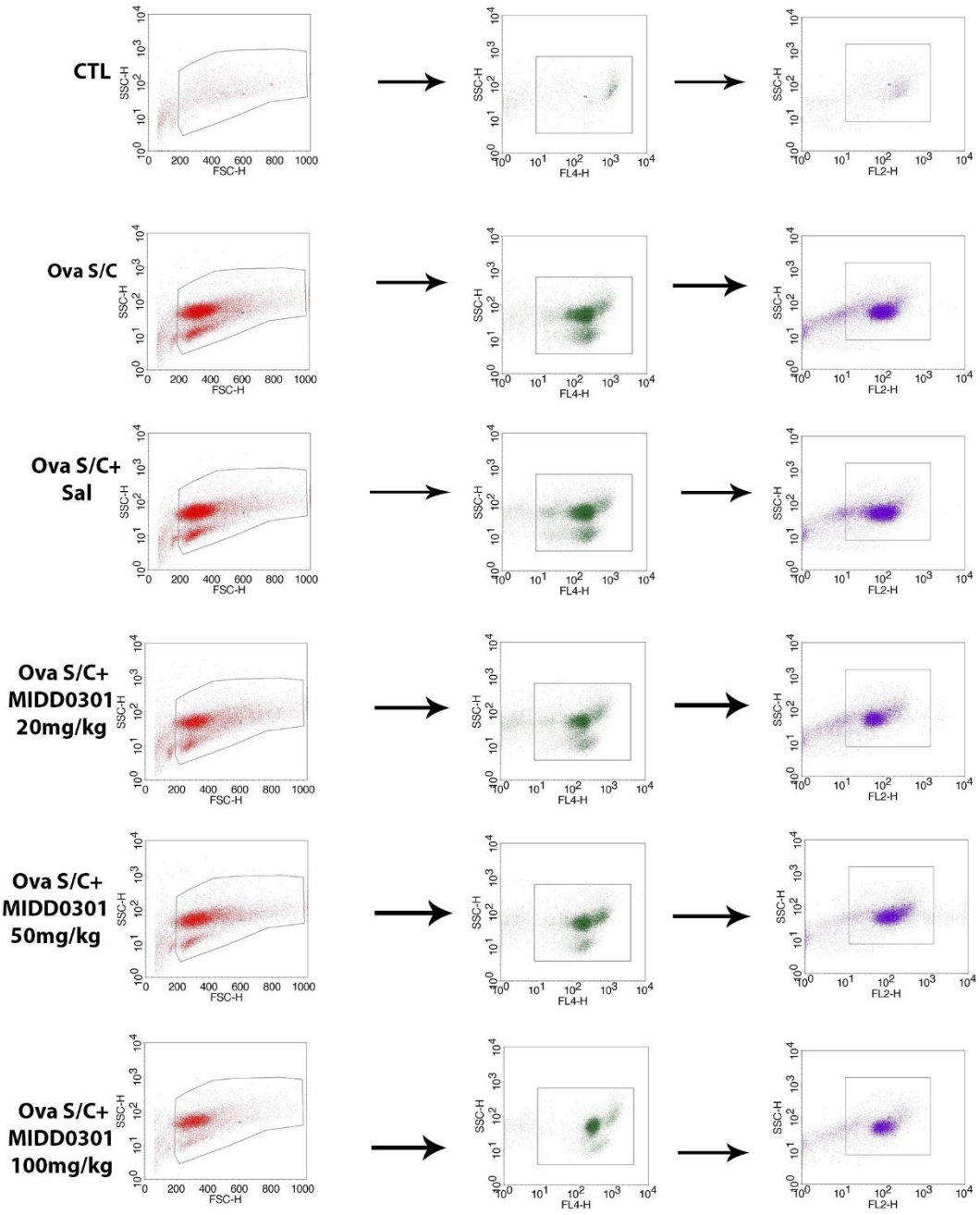

Figure S6. Gating strategy for Siglec F+ cells in BALF of Ova s/c mice administered vehicle or 100 mg/kg, 50 mg/kg, 20 mg/kg of MIDD0301 via oral gavage b.i.d. for 5 days or salmeterol also via oral gavage at 1 mg/kg b.i.d. for 5 days. (FL4-H = CD45 and FL2-H = Siglec F)

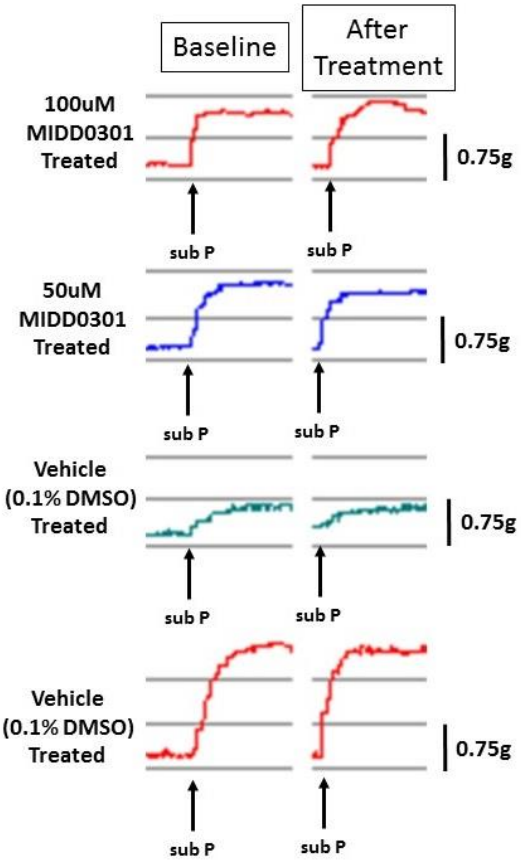

Figure S7. Reversibility of MIDD0301. Representative raw muscle force tracings of guinea pig airway smooth muscle contracted with 1 μM substance P at baseline and after buffer washout of selected treatments. Individual rings were contracted with 1 μM substance P (baseline contraction) and treated with vehicle (0.1% DMSO) or 50 μM or 100 μM MIDD0301. After relaxation had been measured for 1h buffer was changed 9 times over 10 min to washout out treatments and tissues were re-challenged with 1 μM substance P. The magnitude of the substance P contractions were similar before and after treatment supporting the effective washout of the treatment and the retained contractile viability of the tissue.

Mice Pharmacokinetic study. 35 day old female Swiss Webster mice received intra-gastric gavage of vehicle or MIDD0301 formulated in 2% hydroxypropyl methylcellulose and 2.5% polyethylene glycol at a dose of 25 mg/kg. At 10, 20, 40 60,120, 240, 480, and 1440 minutes, the blood (collected into heparinized tubes), lungs and brain were harvested, and samples were stored in liquid nitrogen until analysis.

Sample preparation and LCMS/MS. Blood samples were thawed on ice, vortexed for 10 seconds, and a 100 μL aliquot taken and added to 400 μL cold methanol containing 300 nM compound 2[2] internal standard (I.S.). Samples were vortexed for 30 seconds and centrifuged at 10,000 RPM for 10 minutes. The supernatant layer was then transferred to clean tubes and evaporated using a 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 were properly diluted, 4,5 diphenylimidazole added, and 5 μL of the sample injected into the LC–MS/MS (Shimadzu 8040). Brain and lung tissue samples were thawed, weighed, and

homogenized directly into 400 µL methanol containing compound 2 (I.S.) using a Cole Palmer LabGen 7B Homogenizer. Samples were centrifuged for 10 minutes at 10,000 RPM. The supernatant was then retrieved, and prepared in the same manner as the blood samples for LC-MS/MS analysis (Shimadzu 8040).

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 C_{18} column (2.1 mm × 100 mm, 5 um particle size, Restek, California, US) under gradient elution at a flow rate of 0.6 mL/min. The mobile phase was methanol and water (both containing 0.1% formic acid). Time program: 20% B (0 min) → 70% B (2 min) → 99% B (5 min), hold at 99% B (5.5 min), return to 20% B (5.75 min), hold at 20% B (6 min). Column Temperature: 40°C.

Analytes were monitored under positive mode by Shimadzu 8040 triple quadrupole mass analyzer (Shimadzu, Kyoto, Japan) using electrospray and atmospheric pressure ionization run in dual (DUIS) mode. The following transitions are monitored in multiple reaction monitoring (MRM) mode. Ion pairs for MIDD0301 are m/z 413.90 > 396.00, m/z 413.90 > 368.00, m/z 413.90 > 355.05, m/z 413.90 > 302.95, m/z 413.90 > 326.80, m/z 413.90 > 276.05, m/z 413.90 > 248.20, and m/z 413.90 > 168.10. Transition ion pairs for compound 2 (I.S.) 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 4,5-diphenylimidazole are m/z 220.80 > 193.10, m/z 220.80 > 166.90, m/z 220.80 >151.95 and m/z 220.80 > 116.10. 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: $c = A \bullet e^{-at} + B \bullet e^{-bt} + C \bullet e^{-ct}$.

Rotarod assay: 42 day old female 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 for three consecutive trials. Separate groups of nine mice received oral gavage of vehicle (2% hydroxypropyl methylcellulose and 2.5% polyethylene glycol) or MIDD0301 (100 mg/kg) in a 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, as it is 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.

Liver microsome stability assay. 4 µl of 1 mM test compound (at a final concentration of 10 µM) in DMSO was pre-incubated at 37°C for 5 minutes on a digital heating shaking dry bath (Fischer scientific, Pittsburgh, PA) in a mixture containing 282 µl of water, 80 µl of phosphate buffer (0.5 M, pH 7.4), 20 µl of NADPH Regenerating System Solution A (BD Bioscience, San Jose, CA) and 4 µl of NADPH Regenerating System Solution B (BD Bioscience, San Jose, CA) in a total volume of 391.2 µl. Following preincubation, the reaction was initiated by addition of 8.8 µL of either human liver microsomes (BD Gentest, San Jose, CA) or mouse liver microsomes (Life technologies, Rockford, IL), at a protein concentration of 0.5 mg/ml. Aliquots of 50 µl were taken

at time intervals of 0 (without microsomes), 10, 20, 40, 60, 90 and 120 minutes. Each aliquot was added to 100 µl of cold acetonitrile solution containing 3 µM of 4,5 diphenylimidazole as internal standard. This was followed by sonication for 10 seconds and centrifugation at 10,000 rpm for 5 minutes. 100 µl of the supernatant was transferred into Spin-X HPLC filter tubes (Corning Incorporated, NY) and centrifuged at 13,000 rpm for 5 minutes. The filtrate was diluted 100 fold and analyzed subsequently by LC-MS/MS with the Shimadzu LCMS 8040 instrument). The ratio of the peak areas of the internal standard and test compound was calculated for every time point and the natural log of the ratio were plotted against time to determine the linear slope (k). The metabolic rate ($k*C_0/C$), half-life (0.693/k), and internal clearance ($V*k$) were calculated, where k is the slope, C_0 is the initial concentration of test compound, C is the concentration of microsomes, and V is the volume of incubation in µL per microsomal protein in mg. All experiments represent two independent days in triplet.

Determination of mouse plasma binding. 1 mM MIDD0301 and 500 nM of 4,5 diphenylimidazole were prepared in methanol. Mouse plasma was thawed on ice and 495 µl was dispensed into an Eppendorf tube together with 5 µl of 1 mM MIDD0301. 400 µl of this solution was transferred into the red marked retainer well of a rapid equilibrium dialysis device (Thermo Scientific). 600 µl PBS buffer was added to the adjacent buffer chamber. This was repeated three times at different locations in the plate. The plate was sealed with tape and placed into a shaker for 4 hours at orbital shaking rate of 250 rpm. 50 µl of sample from the red well was transferred to an Eppendorf tube and combined with 50 µl PBS. 50 µl of sample from the buffer well was transferred to an Eppendorf tube and combined with 50 µl mouse plasma. 300 µl of ice cold methanol was added to each of the tubes to precipitate proteins. The tubes were vortexed and incubated 30 minutes on ice followed by centrifugation for 10 min at 10000 rpm. 50 µl of supernatant was added to a new Eppendorf tube and combined with 25 µl of a 500 nM 4,5 diphenyl imidazole solution as ISTD followed by the addition of 425 µl of methanol. The samples were analyzed by LCMS/MS using a Shimadzu 8040 instrument. Quantifications was achieved by using a calibration curve. The assay was repeated three times.

Patch clamp studies with transient transfected cells. Recombinant $GABA_A$ receptors were expressed in transiently transfected HEK-293T cells. Cells were voltage-clamped at -50 mV in the whole-cell recording configuration and GABA or GABA+ MIDD0301 were applied for 5 seconds. The peak current was measured, divided by the response to GABA, and multiplied by 100 to give the % response to GABA alone. The GABA concentration was EC_{3-5} for each subunit combination.[3]

Mammalian $GABA_A$ receptor subunits (human or rat, from Dr. Robert Macdonald, Vanderbilt University and Dr. David Weiss, University of Texas Health Science Center, San Antonio, TX) were transfected using calcium phosphate precipitation as described in Alexeev et al..[4] In addition to 2 µg of each subunit, 1 µg of a plasmid encoding a surface antibody (pHook-1, Invitrogen Life Technologies, Grand Island NY) was included to allow positive identification of transfected cells. Selection for transfected cells was performed 20-52 hours after transfection using antigen-coated magnetic beads.[5]

For patch clamp recordings the external bath solution contained (in mM) 142 NaCl, 8.1 KCl, 6 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) with pH = 7.4 and osmolarity adjusted to 295−305 mOsm. Recording electrodes were filled with a solution of (in mM) 153 KCl, 1 $MgCl_2$, 5 K-EGTA (ethylene glycol-bis(β- aminoethyl ether N,N,N′N′-tetraacetate), and 10 HEPES with pH = 7.4 and osmolarity adjusted to 295−305 mOsm. GABA was diluted into the bath solution from freshly made or frozen stocks in water. MIDD0301

was dissolved in DMSO and diluted into bath solution. Solutions were applied to cells using a 3-barrel glass tube and a computer-driven exchanger (SF-77B, Harvard Apparatus, Holliston, MA, open tip exchange time of <50 ms). Currents were recorded with an Axon 200B (Foster City, CA) patch clamp amplifier and analyzed using the programs Clampfit (Axon Instruments, Foster City, CA) and Prism (Graphpad, San Diego, CA).

References

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