molecular pharmaceutics

Article

pubs.acs.org/molecularpharmaceutics

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

Gloria S. Forkuo,[†] Amanda N. Nieman,[†] Nina Y. Yuan,[†] Revathi Kodali,[†] Olivia B. Yu,[†] Nicolas M. Zahn,[†] Rajwana Jahan,[†] Guanguan Li,[†] Michael Rajesh Stephen,[†] Margaret L. Guthrie,[†] Michael M. Poe,[†] Benjamin D. Hartzler,[†] Ted W. Harris,[†] Gene T. Yocum,[‡] Charles W. Emala,[‡] Douglas A. Steeber,[§] Douglas C. Stafford,[†] James M. Cook,[†] and Leggy A. Arnold*,[†]

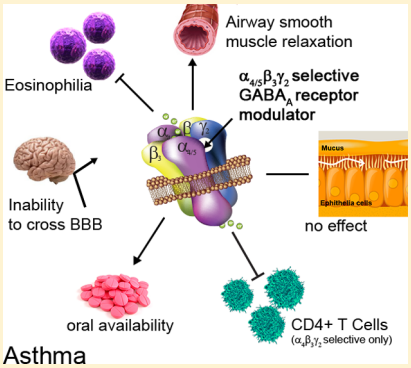
[†]Department of Chemistry and Biochemistry and the Milwaukee Institute for Drug Discovery, University of Wisconsin−Milwaukee, Milwaukee, Wisconsin 53201, United States

[‡]Department of Anesthesiology, Columbia University, New York, New York 10032, United States

[§]Department of Biological Sciences, University of Wisconsin−Milwaukee, Milwaukee, Wisconsin 53201, United States

Supporting Information

ABSTRACT: We describe pharmacokinetic and pharmacodynamic properties of two novel oral drug candidates for asthma. Phenolic $\alpha_4\beta_3\gamma_2$ $GABA_AR$ selective compound **1** and acidic $\alpha_5\beta_3\gamma_2$ selective $GABA_AR$ positive allosteric modulator compound **2** relaxed airway smooth muscle *ex vivo* and attenuated airway hyperresponsiveness (AHR) in a murine model of asthma. Importantly, compound **2** relaxed acetylcholine contracted human tracheal airway smooth muscle strips. Oral treatment of compounds **1** and **2** decreased eosinophils in bronchoalveolar lavage fluid in ovalbumin sensitized and challenged mice, thus exhibiting anti-inflammatory properties. Additionally, compound **1** reduced the number of lung $CD4^+$ T lymphocytes and directly modulated their transmembrane currents by acting on $GABA_ARs$. Excellent pharmacokinetic properties were observed, including long plasma half-life (up to 15 h), oral availability, and extremely low brain distribution. In conclusion, we report the selective targeting of $GABA_ARs$ expressed outside the brain and demonstrate reduction of AHR and airway inflammation with two novel orally available $GABA_AR$ ligands.



KEYWORDS: *asthma, $GABA_A$ receptor, airway hyperresponsiveness, airway inflammation, splenocytes, leukocytes, $\alpha_4\beta_3\gamma_2$, $\alpha_5\beta_3\gamma_2$*

INTRODUCTION

Asthma is a chronic inflammatory lung disorder that affects children and adults of all ages.[1] The prevalence of asthma in the United States continues to rise with an estimated 39.5 million people diagnosed with asthma, including 8.7 million children.[2] Characteristic features of asthma include variable and recurring symptoms of airway inflammation, mucous cell metaplasia, airflow obstruction, and airway hyperresponsiveness (AHR).[3] First line therapy consists of inhaled anti-inflammatory corticosteroids and/or bronchodilators, such as long-acting beta 2 adrenergic receptor agonists, in a stepwise strategy for the long-term management of chronic persistent asthma symptoms.[2] Inhalers are prone to improper use leading to imprecise dosing, oral infections, and overall poor compliance.[4] Severe asthma is treated with systemic corticosteroids, which are associated with adverse side effects such as adrenal suppression, growth suppression, cataract formation, thinning or bruising of the skin, increased mortality, and loss of asthma control.[5−10] Furthermore, orally available leukotriene receptor antagonists are commonly used to manage asthma symptoms;[11] however, up to 78% of patients do not respond to this alternative therapy.[12] Thus, new oral asthma therapies are needed to overcome imprecise drug administration and reduce systemic adverse effects.

The $GABA_A$ receptors ($GABA_AR$) are ligand-gated chloride ion channels best known for their inhibitory role in the central nervous system (CNS). $GABA_ARs$ are heteropentamers assembled from 19 different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, θ, ρ_{1-3}). Classical $GABA_ARs$ consist of two α, two β, and one γ, δ, ε, θ, or π subunit.[13,14] $GABA_AR$ subunits have been identified in airway smooth muscle,[15−18] airway epithelium,[19] and immune/inflammatory cells.[20−24] Evidence that $GABA_ARs$ in the lungs of asthmatic mice can be targeted with small molecules was demonstrated first by Munroe et al.[25] using plant-based honokiol, a positive allosteric $GABA_AR$ modulator with selectivity toward the $\alpha_{1/2/3}\beta_3\gamma_2$ $GABA_AR$ subtypes.[26] The compound reduced AHR and lung eosinophilia upon acute and chronic administration in an ovalbumin sensitized and challenged (ova s/c) mouse asthma model. Allergen challenge in honokiol treated mice demonstrated increased regulatory

Received: March 7, 2017
Revised: April 16, 2017
Accepted: April 25, 2017
Published: April 25, 2017

ACS Publications © XXXX American Chemical Society

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

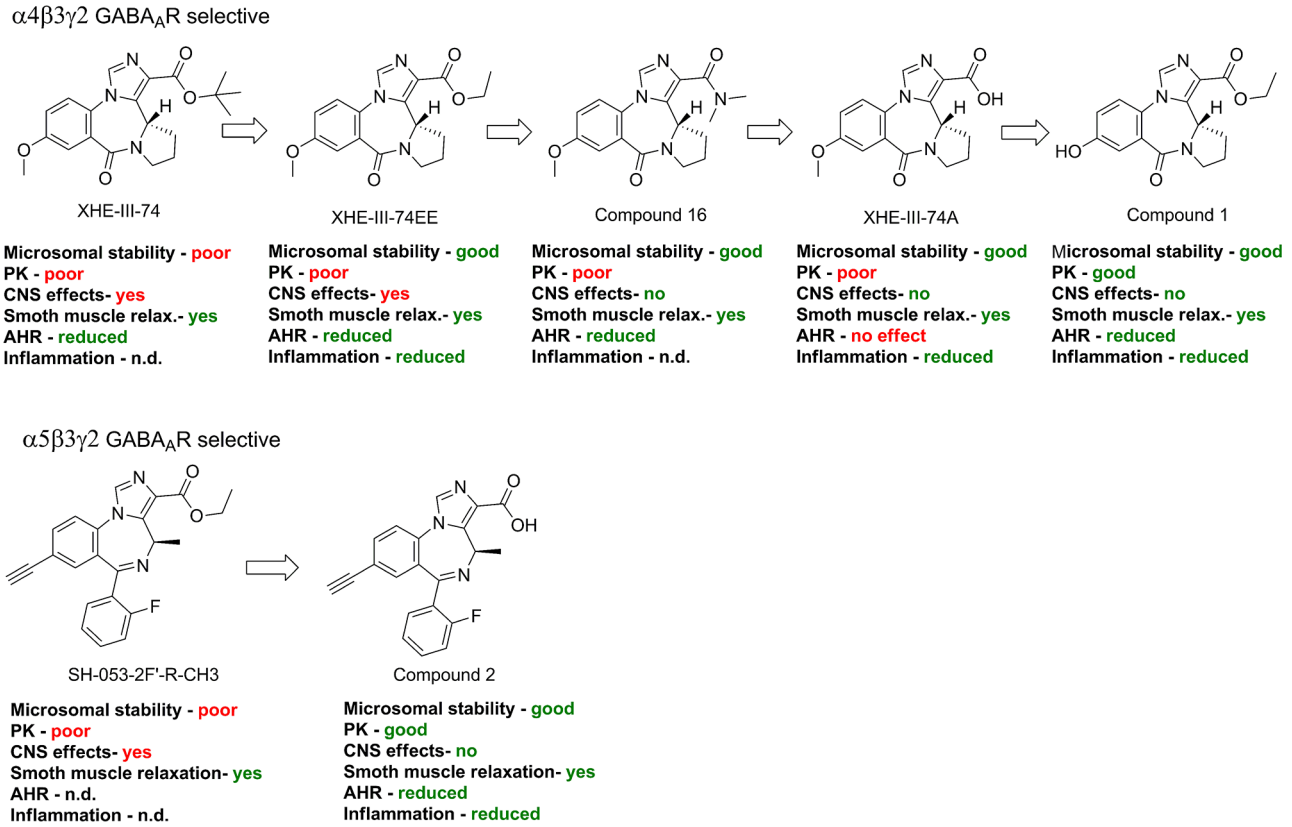


Figure 1. Development of subtype-selective GABA$_A$R modulators as drug candidates for asthma with their most important pharmacological characteristics.

cytokine secretion and reduced pro-inflammatory cytokine release. Selective targeting of the $\alpha_4\beta_3\gamma_2$ GABA$_A$R with XHE-III-74[27] and XHE-III-74EE[23] was shown to alleviate AHR in mouse asthma models (Figure 1).

In addition, these compounds relaxed precontracted rodent and human airway smooth muscle *ex vivo*. Moreover, XHE-III-74A significantly reduced airway eosinophilia in ova s/c mice and reduced IL-2 secretion in phorbol myristate acetate (PMA) and phytohemagglutinin (PHA) activated Jurkat cells.[23] In contrast, AHR alleviating properties of $\alpha_5\beta_3\gamma_2$-selective GABA$_A$R positive allosteric modulators have not been demonstrated, although selective GABA$_A$R ligand SH-053-2′F-R-CH$_3$ has been shown to relax human and guinea pig smooth muscle tissue *ex vivo*.[15,17] XHE-III-74, XHE-III-74EE,[23] and SH-053-2′F-R-CH$_3$[15] can cross the blood−brain barrier (BBB) and induce CNS effects at higher concentrations. In addition, limited pharmacokinetic characterization has been reported for all four compounds.[23,28] In this study, we present the pharmacological properties of compounds **1** and **2** with respect to the pathognomonic features of asthma.

■ EXPERIMENTAL SECTION

Chemicals. Compounds **1** and **2** were synthesized using a published procedure.[41,46] The purity of both compounds (>98%) was confirmed by nuclear magnetic resonance (NMR), high resolution mass spectrometry (HRMS), and liquid chromatography (Supporting Information).

Experimental Animals. Five to ten week old male BALB/c and Swiss Webster mice (Charles River Laboratory, WIL, MA) and adult (425−450 g) male Hartley guinea pigs (Charles River Laboratory, WIL, MA) were used for the experiments. The animals were housed under specific pathogen-free conditions, under standard conditions of humidity, temperature, and a controlled 12 h light and dark cycle and had free access to food and water. All animal experiments were in compliance with the University of Wisconsin−Milwaukee or Columbia University Institutional Animal Care and Use Committees (IACUC).

Ovalbumin Sensitization and Challenge. Randomized male BALB/c mice in a group size of ten were sensitized three times with intraperitoneal (i.p.) injections of 2 mg/kg/d of ovalbumin (ova) (Sigma-Aldrich, St. Louis, MO) emulsified in 2 mg of Alum (Imject Alum; Thermo Scientific, Pierce, Rockford, IL) on days 0, 7, and 14 in a total volume of 100 μL. Mice were anesthetized with isoflurane and challenged intranasally (i.n.) with 1 mg/kg/d ova for 5 days from days 23−27. Control mice were sensitized with ova and challenged with i.n. saline.[47] The chronic effects of compounds **1** and **2** for a duration of 5 days during the ova challenge period were tested in separate groups of ten ova s/c BALB/c mice. As a positive control, separate groups of ten ova s/c BALB/c mice received dexamethasone at 4 mg/kg for 8 days. Airway hyperresponsiveness parameters were assessed on day 28, and mice were sacrificed using an overdose of ketamine/xylazine i.p. on day 29 for assessment of inflammatory cells and mucus metaplasia.

Immunoblot Analysis. Whole spleens from ova s/c BALB/c mice were disrupted and combined with protease inhibitor cocktail tablet (Roche Applied Sciences, Indianapolis, IN) and membrane protein extracted according to the protocol provided by Mem-PER Plus membrane protein extraction kit (Thermo Fisher Scientific Inc., Rockford, IL). Membrane protein concentration was determined by Pierce Coomassie

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

(Bradford) protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL), according to the manufacturer's instructions. Protein extracts were subjected to SDS-PAGE using NuPAGE Novex 4−12% Bis-Tris protein gels (Thermo Fisher Scientific Inc., Rockford, IL) and transblotted onto nitrocellulose iBlot 2 transfer stacks (Thermo Fisher Scientific Inc., Rockford, IL) using the iBlot 2 gel transfer device. Membranes were blocked in superblock T20 (TBS) blocking buffer (Thermo Fisher Scientific Inc., Rockford, IL) for 1 h at room temperature and then incubated with primary antibodies: (α1, dilution 1:100, 06−868, EMD Millipore), (α2, MABN1724, dilution 1:50, EMD Millipore), (α3, dilution 1:200, sc-133603, Santa Cruz), (α4, dilution 1:200, sc-7355, Santa Cruz), (α5, dilution 1:500, sc-7348, Santa Cruz), (β3, dilution 1:500, sc-376252, Santa Cruz), and (γ2, dilution 1:100, MABN875, EMD Millipore) overnight at 4 °C. This was followed by treatment for 1 h with HRP conjugated secondary antibodies: (goat anti-mouse, dilution 1:5000, c-2031, Santa Cruz), (donkey anti-goat, dilution 1:5000, sc-2033, Santa Cruz), and (goat anti-rabbit, dilution 1:5000, sc-2030, Santa Cruz). The protein bands were developed using Pierce ECL Western blotting substrate (Thermo Fisher Scientific Inc., Rockford, IL) according to manufacturer's recommendations. A CCD camera connected to FluorChem HD2/FC2 (Cell Biosciences Inc., Santa Clara, CA) was used to capture the digital images.

Automated Patch-Clamp Electrophysiology for Splenocytes. Splenocytes from four ova s/c BALB/c mice were prepared following BD Biosciences instructions for preparation of murine splenocytes and red blood cells were lysed using BD Pharm Lyse lysing solution (BD Biosciences, San Jose, CA). $CD4^+$ T cells were isolated from splenocytes using an Affymetrix eBiosciences MagniSort mouse $CD4^+$ T cell enrichment kit following manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL). Splenocytes were maintained in suspension in RPMI 1640 medium with L-glutamine (Thermo Fisher Scientific Inc., Rockford, IL) supplemented with 10% (v/v) fetal bovine serum, 10 μM 2-mercaptoethanol and 1% penicillin/streptomycin in the presence or absence of 100 μg/mL ovalbumin. The cells were maintained in 5% CO_2, 95% humidified air at 37 °C for 48 h. Automated patch-clamp studies were conducted as described previously.[23] Briefly, the IonFlux plate layout consists of units of 12 wells: two wells contain intracellular solution (ICS containing 346 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 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose monohydrate, and 10 mM HEPES, pH 7.4 with NaOH), eight contain the compounds of interest diluted in ECS, and one well 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 −50 mV. Prior to use on the automated patch clamp, cells were centrifuged at 380*g* for 2 min and resuspended gently in ECS. This was repeated two more times before the cells were dispensed into the plate. GABA and muscimol were diluted to appropriate concentrations in ECS to appropriate concentrations before application, and data was recorded from eight individual ensembles.

Drug Treatment Protocol. Sterile solutions of compound **1** or **2** were prepared in 2% hydroxypropyl methylcellulose solution (Sigma-Aldrich, St. Louis, MO) and 2.5% polyethylene glycol (Sigma-Aldrich, St. Louis, MO) in a biological safety cabinet. A fine suspension was obtained by grinding the mixture with a mortar and pestle. Drugs were administered individually at 100 mg/kg by oral gavage with 20G gavage needles (Kent Scientific Corporation, Torrington CT) to groups of ova s/c BALB/c twice daily for 5 days during the ova challenge period. Mice received a single p.o. dose of the compounds just before airway parameter measurements. Dexamethasone (Sigma-Aldrich, St. Louis, MO) was prepared in 10% DMSO, 40% propylene glycol, and 50% PBS given as i.p injection. Dexamethasone was administered (4 mg/kg) daily for 8 days. Mice were monitored daily after drug administration.

Assessment of Airway Hyperresponsiveness. Airway hyperresponsiveness to methacholine in conscious, spontaneously breathing animals was measured by DSI's Buxco FinePointe Non-Invasive Airway Mechanics (NAM) instrument.[48] Before measurements were taken, mice were acclimated to the chambers 15 min daily for 5 days. The chambers were also calibrated each time before data collection. Briefly, the nasal chamber in combination with the thoracic chamber allows the computation of specific airway resistance (sRaw). The FinePointe software computes specific airway resistance with all other ventilatory parameters derived by the NAM analyzer. Mice were exposed to aerosolized PBS (for the baseline measurement) or methacholine (1.5625−12.5 mg/mL) for 1 min and readings were taken and averaged for 3 min after each nebulization. Data obtained were presented as sRaw versus the methacholine concentration (mg/mL) used to generate the aerosol.[23]

Eosinophil Count. Immediately after assessment of airway hyperresponsiveness, mice were euthanized with a cocktail of ketamine (300 mg/kg) and xylazine (30 mg/kg) (Sigma-Aldrich, St. Louis, MO) in an approximate volume of 100 μL per i.p. injection. The lungs were dissected and tracheostomized with an 18G luer stub adapter. The left lung was clamped using a hemostat and the right lung perfused with 500 μL sterile saline. Broncho-alveolar lavage fluid (BALF) was then pooled and aspirated into tubes.[23,47]

BALF was spun onto positively charged slides using Thermo Shandon Cytopsin 3 (Marshal Scientific, Brentwood, NH). Slides were stained with Wright Geimsa stain (Sigma-Aldrich, St. Louis, MO) and washed with ddH_2O for 10 min. The slides were coverslipped with Permount mounting media (Fisher Scientific, Pittsburgh, PA) and the number of eosinophils counted using the Labomed TCM 400 inverted microscope light microscope (Labo America Inc., Freemont, CA) in four randomly chosen fields of view at 20×. Eosinophils were determined as a percentage of the total cells in each field and expressed as percent eosinophil.[23,47]

Flow Cytometry. Bronchoalveolar lavage (BAL) was performed with 1 mL of Ca^{2+}- and Mg^{2+}-free PBS. Red blood cells (RBCs) were lysed using BD red blood cell lysis buffer (BD Pharmingen, San Jose, CA). BALF was split into four different tubes and nonspecific binding to Fc receptors was blocked for 5 min using 6 μg/mL of 2.4G2 mouse BD Fc Block (BD Pharmingen, San Jose, CA). BALF cells were stained for 30 min at 4 °C in the dark with 100 μL of BSA stain buffer (BD Pharmingen, San Jose, CA) containing the final concentrations

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

of the following antibodies: antimouse CD45 APC (1:1000, 30-F11, Affymetrix eBiosciences, San Diego, CA), FITC rat antimouse CD4 (1:500, RM4−5, BD Pharmingen, San Jose, CA), FITC rat antimouse CD11b (1:200, M1/70, BD Pharmingen, San Jose, CA), FITC rat antimouse Ly-6G and LY-6C (GR-1) (1:500, RB6−8C5, BD Pharmingen, San Jose, CA), and mouse CCR3 PE-conjugated antibody (1:40, 83101, R&D systems Inc., Minneapolis, MN). Flow cytometric studies were done using the BD FACS Calibur (BD Pharmingen, San Jose, CA) and data analyzed subsequently using Cell Quest pro software (BD Pharmingen, San Jose, CA).[49] Gating strategies for the different markers and treatment groups are shown in Supporting Information (Figures S6−S9). Total inflammatory cell count was obtained by running all samples on high (60 μL/min) for 180 s. The gated anti-mouse CD45 positive events in the fourth channel (FL4) were used to calculate the total inflammatory cell count as cells/mL. The frequencies of $CCR3^+$, $GR1^+$, $CD4^+$, and $CD11b^+$ cell populations in their respective gates were multiplied by the total inflammatory cell count (cells/mL) to obtain the differential cell population.

Statistical Analysis. Data were analyzed using GraphPad Prism 4 (GraphPad Software, San Diego, CA) and expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Dunnet post hoc test or two-way ANOVA with Bonferroni post hoc test were performed for statistical difference for multiple groups. For comparison of two groups, a two-tailed unpaired Student's *t* test was used. Statistical significance was defined as $p < 0.05$.

RESULTS

$GABA_A$Rs Are Expressed in Splenocytes and Respond to GABA and Muscimol. $GABA_A$Rs have been identified in multiple extra-CNS tissues such as the lungs, kidneys, endocrine organs, heart, ovary, testis, placenta, uterus, pancreas, small intestines, and testis.[16,17,23,29,30] Herein, we determined the expression of $GABA_A$R subunits in immune cells collected from the spleen (Figure 2).

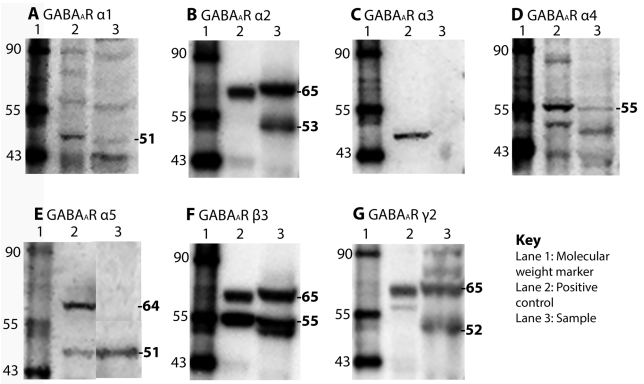


Figure 2. Immunoblot analysis of $GABA_A$ receptor subunits in splenocytes.

Representative immunoblots (A−G) from mouse spleen homogenates of ova s/c BALB/c mice were generated with $GABA_A$R subunit antibodies and 50 μg of membrane protein harvested from the spleen. Lane 1, molecular weight marker (kDa); lane 2, positive controls (α_1, α_3, α_5, β_3, mouse brain extract; α_4, H4 cell lysate; α_2 and γ_2, mouse cerebellum extract); lane 3, membrane protein extracted from spleens of ova s/c BALB/c mice.

Western blotting identified immunoreactive bands for the α_1, α_2, α_4, α_5, β_3, and γ_2 subunits in membrane proteins obtained from spleen tissues of ova s/c BALB/c mice. An immunoreactive band at 51 kDa for α_1 was observed as reported.[31] For α_2, an immunoreactive band at 65 kDa was observed for splenocytes as well as mouse cerebellum extract in addition to the predicted immunoreactive band at 53 kDa. Both bands were described in an earlier report for ova s/c mouse lung extract.[23] The expression of the α_3 $GABA_A$R subunit was not identified. For α_5, a single molecular weight band at 51 kDa was observed for splenocyte extract (Figure 2E). An additional immunoreactive band at 64 kDa was detected for mouse brain extract and ova s/c mouse lung homogenate.[23] The γ_2-specific antibody produced immunoreactive bands at 65 and 52 kDa in splenocytes. A single band at 65 kDa was observed in mouse cerebellum extract. Finally, the immunoreactive bands observed with β_3- and α_4-specific antibodies were identical in splenocyte extract, mouse cerebellum extract, and H4 cell lysate controls (Figure 2D,F). Thus, we confirmed the presence of $GABA_A$R subunits in a heterogeneous immune cell population, which prompted us to investigate the modulation of these receptors by $GABA_A$R agonists. Accordingly, splenocytes from ova s/c mice cultured with and without ovalbumin were investigated by patch clamp. The electrophysiological responses of both cell preparations in the presence of increasing concentration of GABA and $GABA_A$R agonist muscimol were detected at a holding potential of −50 mV (Figure 3).

Patch-clamp is usually carried out on a single cell, making it difficult to investigate a heterogeneous cell population such as splenocytes. However, an automated patch clamp approach using the Ionflux system overcomes this limitation by trapping 20 cells simultaneously and recording an average change of current at a constant holding potential. Using this instrument, we were able to record a change of current at a holding potential of −50 mV for splenocytes in the presence of GABA. This current change was concentration-dependent between 0 and 33 μM GABA with a calculated EC_{50} value of 2.05 μM (Figure 3A,C). Splenocytes from ova s/c mice cultured with or without ovalbumin gave similar current changes. The $GABA_A$R agonist muscimol exhibited a similar affinity to $GABA_A$Rs expressed on splenocytes based on similar EC_{50} values in comparison to GABA (Figure 3B). However, the induction of current alteration was only around 50% of the current change induced by GABA resulting in a lower efficacy of muscimol toward $GABA_A$Rs on splenocytes (Figure 3C,D).

Compounds 1 and 2 Relax Airway Smooth Muscle. The ability of compounds **1** and **2** to relax airway smooth muscle was investigated using two *ex vivo* model systems (Figure 4). These include the application of substance P to guinea pig tracheal rings, causing smooth muscle constriction mediated by the Gq-coupled neurokinin receptors[32] and acetylcholine applied to human tracheal airway smooth muscle strips, acting at muscarinic acetylcholine receptors that include Gi and Gq-protein coupled receptors.[33]

Compound **1** significantly reduced substance P-induced contraction of guinea pig ASM at 15, 30, 45, and 60 min (Figure 4A). The contractile force induced by substance P spontaneously decreased over a period of 1 h; however, significant smooth muscle relaxation was observed for 50 μM compound **1** at each time point. The response was concentration dependent (Figure 4B). In contrast, acetylcholine induced a long lasting smooth muscle constriction in human tracheal airway smooth muscle strips that did not

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

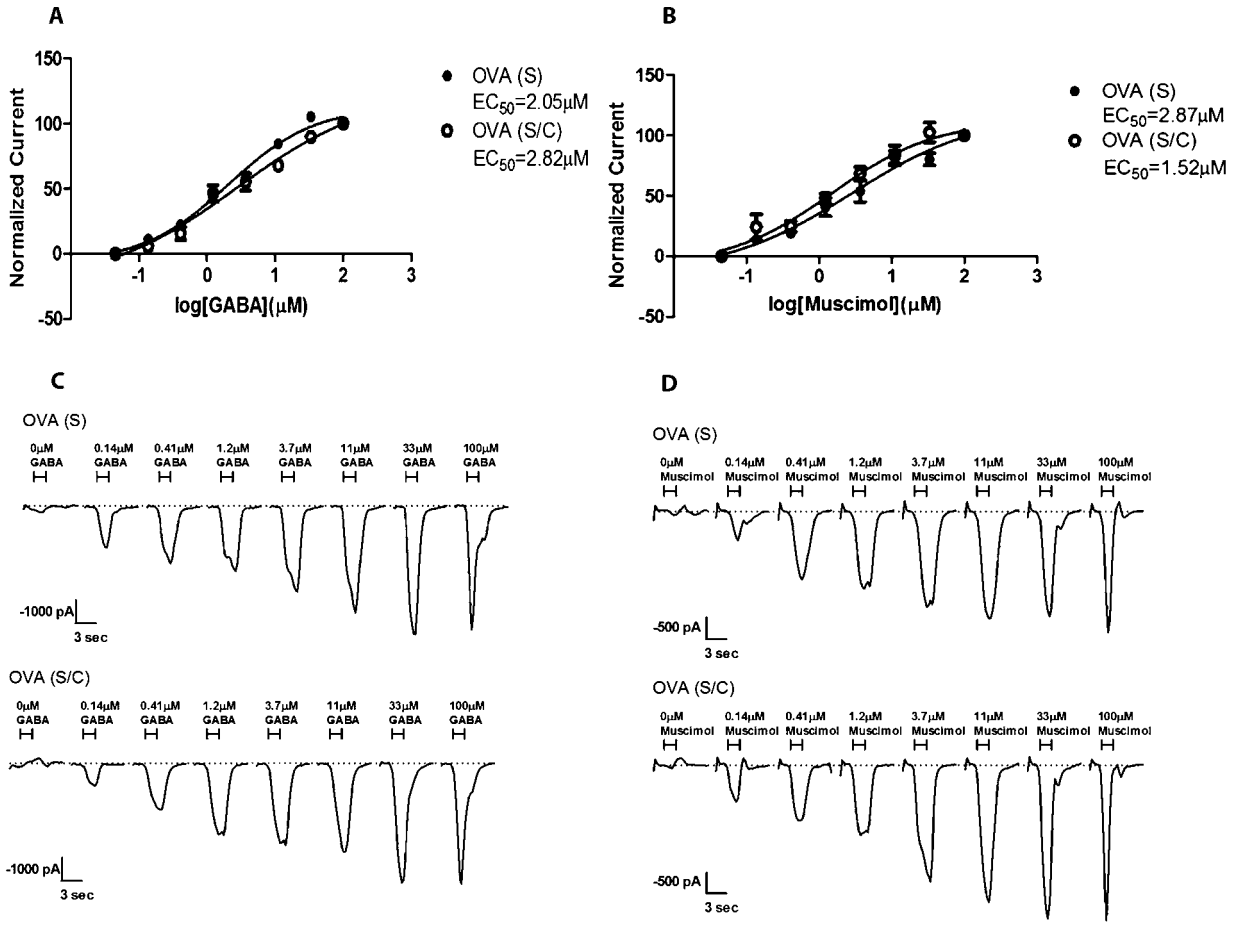


Figure 3. Electrophysiological response of splenocytes in the presence of $GABA_AR$ agonists. (A) Concentration-dependent current responses of splenocytes from ova s/c BALB/c mice cultured with (s/c) and without (s) ovalbumin in the presence of GABA normalized to the highest current change (n = 8). (B) Concentration-dependent current responses of splenocytes from ova s/c BALB/c mice cultured with (s/c) and without (s) ovalbumin in the presence muscimol normalized to the highest current change (n = 8). (C) Current recordings of different concentrations of GABA applied for 3 s using splenocytes from ova s/c BALB/c mice cultured with (s/c) and without (s) ovalbumin. (D) Current recordings of different concentrations of muscimol applied for 3 s using splenocytes from ova s/c BALB/c mice cultured with (s/c) and without (s) ovalbumin.

change during 1 h (Figure 4C). For compound **2** at 100 μM, a time-dependent relaxation of constricted human tracheal airway smooth muscle was observed. The smooth muscle relaxation was significant after 15 min. In addition, the concentration of compound **2** in the human airway smooth muscle was quantified after a 1 h incubation with 100 μM of compound **2**. In contrast to the bath concentration of 100 μM compound **2**, the concentration of compound **2** in tissue after extraction and quantification by LC−MS/MS was 9.8 μM. When applying different concentrations (Figure 4D), we observed that 50 μM compound **2** was sufficient to relax guinea pig smooth airway muscle after 30 min.

Compounds 1 and 2 Delivered Orally in Mice Distribute Readily to Lung but Not Brain. Pharmacokinetic analysis of drug candidates is essential to ensure pharmacological concentrations in target tissues and enables further dosage optimization. The pharmacokinetic profiles of compounds **1** and **2** are presented in Figure 5.

Pharmacokinetic analysis following oral administration of compound **1** at 25 mg/kg indicated absorption within 1 h and excellent distribution (AUC = 2270.2 μg·min/g) with a T_{max} of 60 min in blood and lungs (Figure 5A). The C_{max} was highest in blood followed closely in lungs with a C_{max} of 10.23 μg/g. The half-life of compound **1** was shorter in blood than in lungs (238 min), as compound **1** was still detected in lungs after 8 h (Figure 5A). Importantly, extremely low amounts of compound **1** were observed in brain. Compound **2** exhibited pharmacokinetic properties similar to compound **1**. The acid was well absorbed and distributed with a T_{max} of 60 min in blood and lungs (Figure 5B). Comparable concentrations of compound **2** were observed in blood and lungs. However, compound **2** had a significantly longer half-life in the blood (∼15 h) compared to compound **1**. Like compound **1**, compound **2** was still detected after 8 h in lung tissue. Most importantly, negligible concentrations of compound **2** were detected in the brain. At a dosage of 100 mg/kg, compounds **1** and **2** were given orally to mice to investigate any CNS effect when balancing on a rotating rod (rotarod assay). As presented in the SI Figure 1, no motor sensory impairment was observed. Furthermore, low efficacy toward the $\alpha_1\beta_3\gamma_2$ $GABA_AR$, which mediates sedation and tolerance, was demonstrated for compound **1** (SI Figure 2) and compound **2**.[34]

Compounds 1 and 2 Have Anti-Inflammatory Properties. Flow cytometry was used to quantify the changes of inflammatory cells in bronchoalveolar lavage fluid (BALF) of ova s/c BALB/c mice when treated orally with compound **1** or **2** at doses of 100 mg/kg twice daily for 5 days (Figure 6A−F).

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

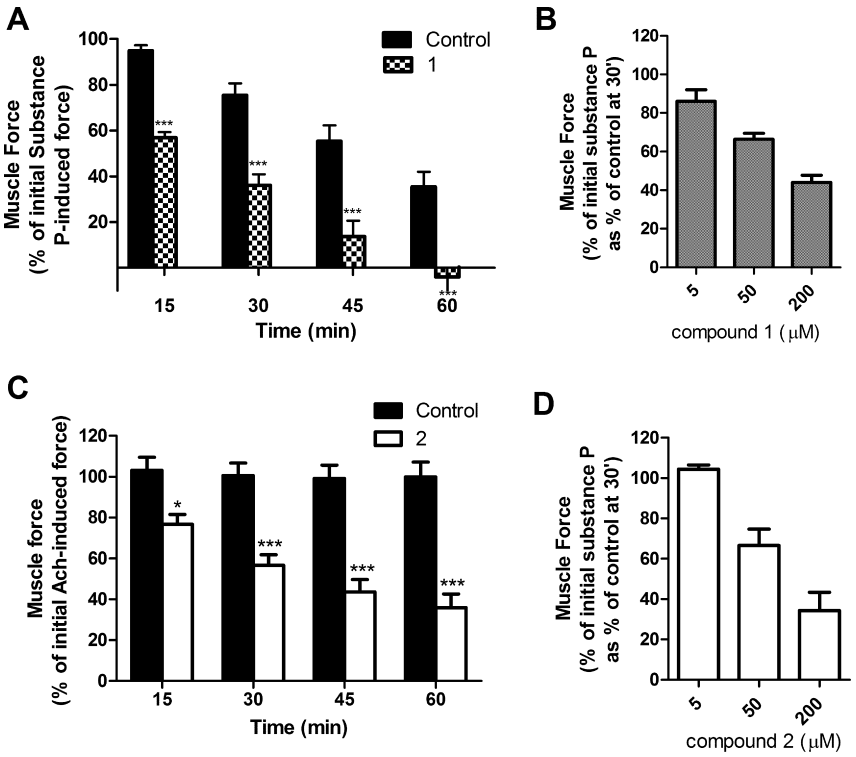
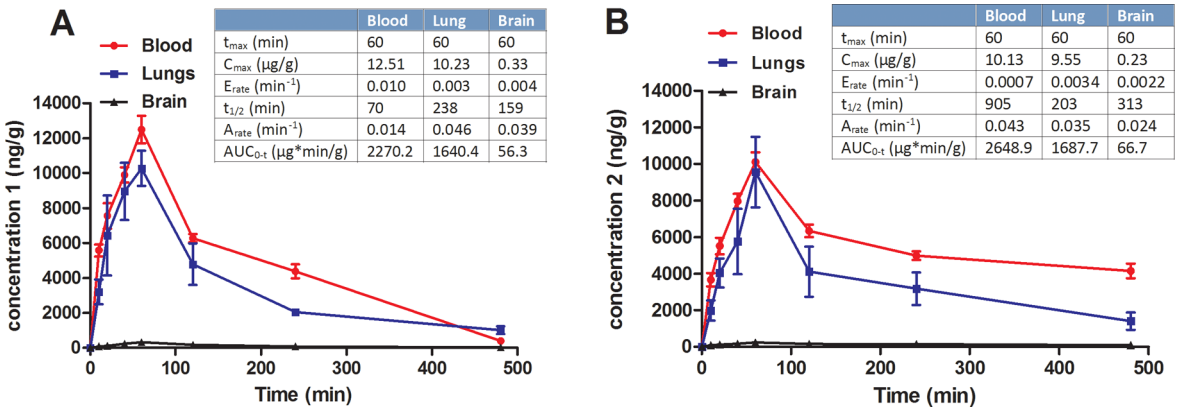


Figure 4. Smooth muscle contractile force measurement in the presence of compound **1** or **2**. (A,B) Airway smooth muscle contractile force in guinea pig tracheal rings. Tracheal rings were contracted with 1 μM substance P and then treated with (A) 50 μM compound **1** (or the vehicle control 0.1% DMSO). The percent of remaining contractile force was measured at various time points and expressed as a percent of the initial substance P induced contractile force ($N = 4$). (B) Tracheal rings were contracted with 1 μM substance P and then treated with compound **1** at different concentrations. The percent of remaining contractile force was measured at 30 min and expressed as a percent of the initial substance P induced contractile force as a percent of control ($N = 3$) (C) Human tracheal airway smooth muscle strips were contracted with an EC_{50} concentration of acetylcholine (Ach) and then treated with 100 μM of compound **2** or the vehicle 0.2% ethanol. Muscle force was measured at 15, 30, 45, and 60 min after addition of compound **2**. Data are expressed as the percent of the initial Ach-induced contractile force. Individual muscle strips from at least seven humans were used. (D) Guinea pig tracheal rings were contracted with 1 μM substance P and then treated with different concentrations of compound **2**. The percent of remaining contractile force was measured at 30 min and expressed as a percent of the initial substance P-induced contractile force as a percent of control ($N = 3$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the vehicle control.



	Blood	Lung	Brain
t_{max} (min)	60	60	60
C_{max} (μg/g)	12.51	10.23	0.33
E_{rate} (min^{-1})	0.010	0.003	0.004
$t_{1/2}$ (min)	70	238	159
A_{rate} (min^{-1})	0.014	0.046	0.039
AUC_{0-t} (μg*min/g)	2270.2	1640.4	56.3

	Blood	Lung	Brain
t_{max} (min)	60	60	60
C_{max} (μg/g)	10.13	9.55	0.23
E_{rate} (min^{-1})	0.0007	0.0034	0.0022
$t_{1/2}$ (min)	905	203	313
A_{rate} (min^{-1})	0.043	0.035	0.024
AUC_{0-t} (μg*min/g)	2648.9	1687.7	66.7

Figure 5. Pharmacokinetic profile of compounds **1** and **2** in mice blood, lungs, and brain. (A) Time-dependent systemic distribution of compound **1** (25 mg/kg via oral gavage) and (B) compound **2** (25 mg/kg via oral gavage).

Leukocytes were incubated with fluorescent mouse CD45 antibody, which is specific for protein tyrosine phosphatase that is selectively expressed in hematopoietic cells. Ova s/c mice that exhibit asthma-like inflammation showed a significant increase of white blood cell numbers in BALF (Figure 6A). Importantly, oral administration of compounds **1** and **2** for 5 days decreased the number of leukocytes in BALF, demonstrating anti-inflammatory properties. The reduction of white blood cells was more pronounced for i.p. administration of 4 mg/kg dexamethasone over an 8 day treatment course. Eosinophilia in BALF, a hallmark of inflammation in ova s/c mice,[35] was quantified by selective staining using Wright Geimsa (Figure 6B). Treatment of compound **1** ($p < 0.001$) or **2** ($p < 0.05$) for 5 days b.i.d. significantly decreased eosinophils in mouse BALF. To further differentiate the change of mouse BALF leukocyte populations, flow cytometry using cell type-specific fluorescent

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

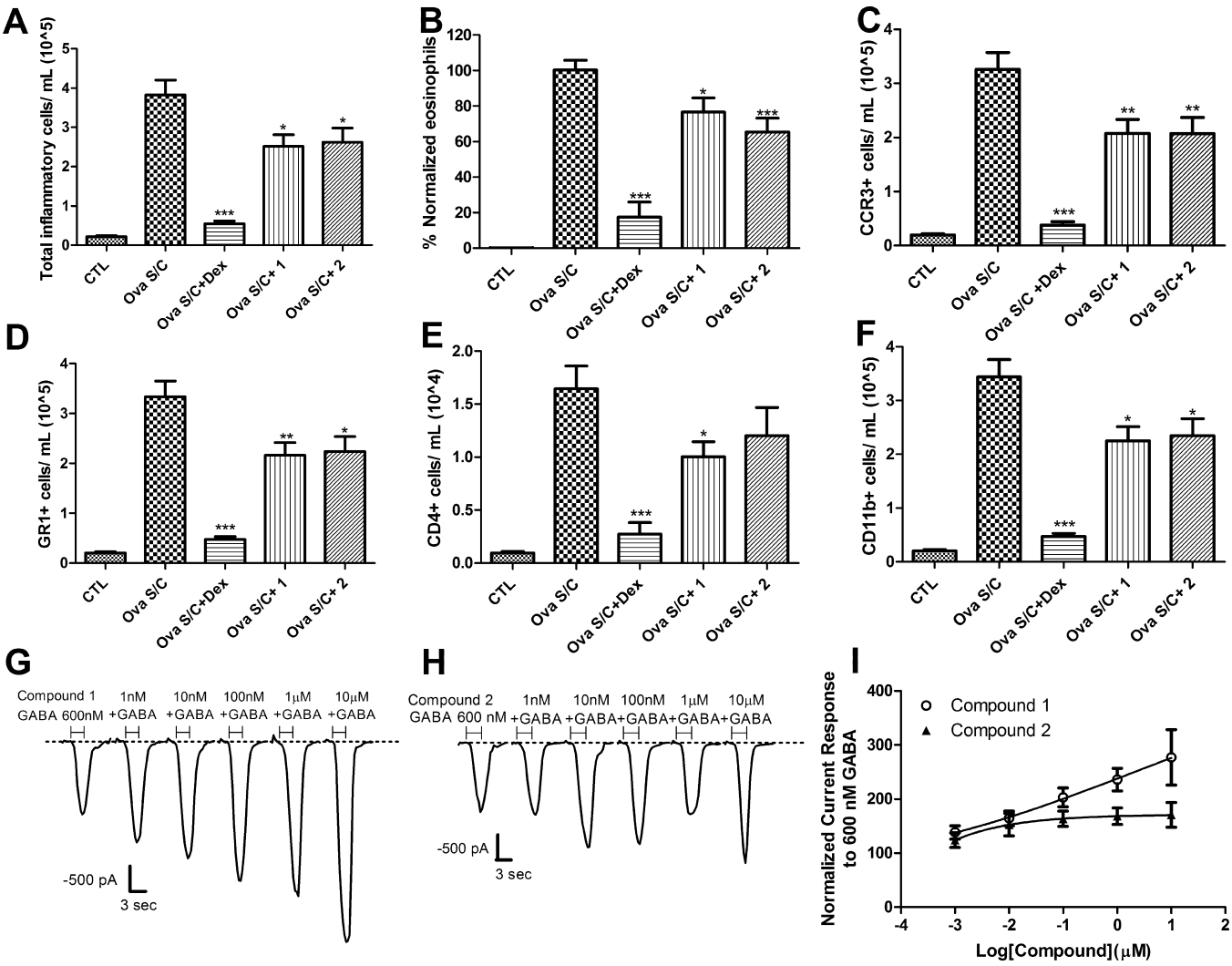


Figure 6. Effect of compounds **1** and **2** on inflammatory cells. Groups of 10 ova s/c BALB/c mice were administered dexamethasone i.p., 4 mg/kg daily for 8 days; compound **1** via oral gavage, 100 mg/kg twice daily for 5 days, and compound **2** via oral gavage, 100 mg/kg twice daily for 5 days. BALF was harvested from each animal and used for (A) quantification of total inflammatory cells. Cells were stained with mouse CD45 APC antibody, and samples were analyzed with BD FACS Calibur on high flow rate (60 μL/min) for 180 s. The gated positive events in the fourth channel (FL4) as depicted in the Supporting Information were used to calculate the total inflammatory cell count as cells/mL. (B) Quantification of Wright Giemsa stained airway eosinophils. Data represent % normalized eosinophil count relative to CTL (negative control) and ova s/c mice (positive control). Examples of captured pictures are presented in Supporting Information (SI Figures 5−8). (C−F) Quantification of specific leukocyte population. (C) CCR3$^+$, (D) GR1$^+$, (E) CD4$^+$, and (F) CD11b$^+$ cell populations were stained with specific antibodies and detected by flow cytometry. Data represent mean ± SEM from 10 mice in each group. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ significance, respectively, compared to vehicle treated ova s/c BALB/c mice. (G) Current recordings in the presence of 600 nM GABA and increasing concentration of compound **1** applied together for 3 s using CD4$^+$ T-cells isolated from ova s/c BALB/c mice spleen. (H) Current recordings in the presence of 600 nM GABA and increasing concentrations of compound **2** applied together for 3 s using CD4$^+$ T-cells isolated from ova s/c BALB/c mice spleen. (I) Concentration-dependent current responses of CD4$^+$ T-cells from ova s/c BALB/c mice spleen in the presence of 600 nM GABA and increasing concentration of compound **1** or **2**. Current readings were normalized to 600 nM GABA response set as 100% (n = 16).

antibodies was carried out. Eosinophil quantification was achieved by immunostaining with a fluorescent antibody to chemokine receptor type 3 (CCR3, CD193). As demonstrated in Figure 6B, BALF from ova s/c mice contained a large number of eosinophils that were significantly reduced by oral treatment with compound **1** or **2** (Figure 6C). Dexamethasone i.p. treatment almost completely eliminated the presence of eosinophils. A similar study using fluorescent GR1 antibody, selective for mouse Ly-6G/Ly-6C myeloid differentiation antigens expressed on granulocytes and macrophages, mirrored these results (Figure 6D). Quantification of CD4$^+$ helper T lymphocytes was achieved by fluorescent anti-CD4 immunostaining. In comparison to granulocytes, a relatively smaller number of CD4$^+$ T cells were detected in BALF of ova s/c mice (Figure 6E). Although 5 day oral treatment of compound **2** b.i.d. significantly reduced the number of eosinophils, a similar significant reduction in CD4$^+$ T cells was not observed. However, the same treatment regimen with compound **1** significantly reduced both of CD4$^+$ T cell and eosinophil numbers. As expected, dexamethasone treatment at 4 mg/kg i.p. for 8 days significantly reduced the CD4$^+$ T cell population. Finally, neutrophils, basophils, monocytes, and macrophages were quantified, as detected by immunostaining with fluorescent CD11b antibody, selective for integrin alpha M

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

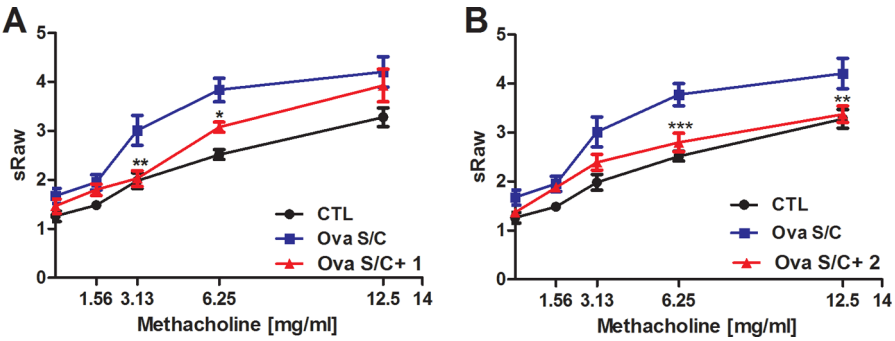


Figure 7. Effect of compounds **1** and **2** on airway hyperresponsiveness. Specific airway resistance (sRaw) was measured at increasing dosages of methacholine by a DSI's Buxco FinePointe noninvasive airway mechanics instrument. Ova s/c BALB/c mice were administered (A) compound **1** via oral gavage, 100 mg/kg twice daily for 5 days or (B) compound **2** via oral gavage, 100 mg/kg twice daily for 5 days. Data represent mean ± SEM from 10 mice in each group. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ significance, respectively, compared to vehicle treated ova s/c BALB/c mice.

(Itgam, Mac-1, CR3A, CD11b). For ova s/c mice, a significant increase in this leukocyte population was observed in BALF. Importantly, treatment with compound **1** or **2** significantly reduced their number in the BALF of ova s/c mice, as did dexamethasone treatment (Figure 6F). In addition, $CD4^+$ T cells were isolated from spleen of ova s/c mice and their change of transmembrane currents was determined by patch clamp in the presence of compounds **1** and **2** and low concentrations of GABA. Compound **1** induced a concentration-dependent increase in transmembrane current (Figure 6G). The modulation of transmembrane currents was not concentration dependent for compound **2** (Figure 6H). Overall, the electrophysiological effect on $CD4^+$ T cells was more pronounced for compound **1**, which induced large current changes at concentration higher than 1 μM (Figure 6I).

Compounds 1 and 2 Reduce Airway Hyperresponsiveness. AHR is a cardinal feature of asthma, characterized by physiologic perturbation to cholinergic agonists.[36] Pharmacodynamic properties of orally administered compound **1** or **2** were investigated with ova s/c mice in the presence of increasing concentrations of aerosolized methacholine by measuring specific airway resistance (sRaw) with a Buxco FinePointe noninvasive airway mechanics instrument (Figure 7A,B).

Significant reduction of AHR was observed at methacholine concentrations of 6.25 and 12.5 mg/mL. Administration of compound **1** (5 days, 100 mg/kg b.i.d.) including 30 min before the measurement caused significant reduction of sRaw values at 3.13 and 6.25 mg/mL methacholine challenge doses (Figure 7A). These results are in agreement with results published previously with α_4-selective $GABA_AR$ ligand XHE-III-74EE that protected against AHR at low methacholine concentrations.[23] The report also demonstrated the reduced AHR for dexamethasone treated ova s/c mice. Importantly, administration of compound **2** produced a significant downward shift of sRaw values and therefore protection of AHR at high methacholine concentrations (6.25 and 12.5 mg/mL methacholine) (Figure 7B). The mouse lung was investigated further using periodic acid fluorescent Schiff's stained sections to assess mucus content (Figure S4). Oral administration of compound **1** or **2** did not significantly change mucous metaplasia compared to vehicle-treated ova s/c mice.

■ DISCUSSION

Inhaled corticosteroids and beta 2 adrenergic receptor (β_2AR) agonists are cornerstones of the long-term management of moderate to severe persistent asthma.[37,38] Although the inhaled route of administration offers targeted drug delivery of high dosages to the lung and reduced systemic side effects (such as osteoporosis, impaired immunity, and reduced growth associated with the prolonged use of corticosteroids), it poses drug adherence and compliance issues for patients.[39,40] Inhaled asthma therapeutics can also be expensive and inconvenient and some have to be carried at all times.[39] Hence, both children and the elderly find this route of drug administration difficult. Poor inhalation technique greatly affects drug delivery to the lungs and is the principal reason for poor medication efficacy. This subsequently affects the outcome of asthma therapy resulting in increased morbidity and mortality.[39,40] Oral administration, however, is widely accepted and can be supervised by parents and caregivers. Furthermore, long-lasting drug exposure and resulting pharmacological effects enable protection during day and night.

Here, we demonstrate the pharmacological effects of novel $GABA_AR$ positive allosteric modulators that, unlike commonly known ligands with such activity, have extremely low distribution in brain. Low brain exposure was confirmed by no detectable adverse CNS effects, as measured with a murine sensorimotor assay at high oral dosages (Figure S1). The innovative chemical compositions include hydrophilic groups such as phenolic hydroxyl (compound **1**) and carboxylic acid (compound **2**) attached to well-known imidazobenzodiazepine scaffolds. Although different in their acidity (pK_A), both changes almost completely reduced brain exposure. Importantly, compounds **1** and **2** exhibited excellent pharmacokinetics with very high concentrations in blood and lung. A long *in vivo* half-life, especially observed for compound **2** (15 h) would ensure protection against asthma exacerbation during the day and when taken again during the night. This feature is similar to long acting β-agonists. We observed that oral b.i.d. administration of compound **1** for 5 days during the ovalbumin challenge period significantly attenuated AHR at low concentrations of methacholine. This result is in agreement with previously investigated α_4-selective positive allosteric $GABA_AR$ modulator XHE-III-74EE administered for 7 days via osmotic pump.[23] In addition, chronic i.p. administration of 20 mg/kg XHE-III-74EE or 40 mg/kg of its dimethylamide analogue b.i.d. for 5 days reduced sRaw values at the highest

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

methacholine concentration used.[23,41] Similar results were observed in a house dust mite model with C57BL/6 mice with XHE-III-74 when administered as aerosol 10 min prior to the AHR measurement.[27] Thus, $GABA_AR$ ligands derived from the same scaffold with significant selectivity toward the $\alpha_4\beta_3\gamma_2$ $GABA_AR$ have been shown to attenuate AHR when administered by different routes and variable dosages. Furthermore, we demonstrated for the first time that compound **2**, an $\alpha_5\beta_3\gamma_2$ $GABA_AR$ selective positive modulator given orally b.i.d. at 100 mg/kg, protected against AHR.

The *in vivo* effects of compounds **1** and **2** on airway smooth muscle relaxation mimicked the *ex vivo* airway smooth muscle effects. In organ bath experiments, compounds **1** and **2** significantly relaxed substance P mediated precontraction of guinea pig airway smooth muscle. Similar pharmacological effects of selective $\alpha_4\beta_3\gamma_2$ or $\alpha_5\beta_3\gamma_2$ positive allosteric $GABA_AR$ modulators have been reported previously.[15,17,23,27,34] Importantly, compound **2** was additionally efficacious in a clinically relevant model where human tracheal smooth muscle was exposed to the contractile mediator acetylcholine, which in addition to muscle constriction mediates airway inflammation and mucus hypersecretion.[42] The actual concentration of compound **2** in the tissue was 9.8 μM, although the concentration in the organ bath was 100 μM. Thus, single digit micromolar concentrations of $GABA_AR$ modulators enabled airway muscle relaxation.

Another important pharmacological property of the selective positive allosteric $GABA_AR$ modulators tested herein is their absence of mucogenic properties (Figure S4). A recent study has shown an excitatory rather than an inhibitory GABAergic system in epithelium, whose inhibition decreased mucous production.[19] However, compounds that specifically positively modulate the $\alpha_4\beta_3\gamma_2$ or $\alpha_5\beta_3\gamma_2$ $GABA_AR$ subtype did not increase mucus production in ova s/c mice.[23]

Airway inflammation is one of the hallmarks of asthma. It can be treated successfully with inhaled corticosteroids such as fluticasone,[43] resulting in fewer side effects than oral corticosteroids such as dexamethasone or prednisolone, causing among others cardiovascular[44] and CNS effects.[45] To develop a single compound to combat AHR and airway inflammation, we target $GABA_AR$ proteins expressed on immune/inflammatory cells that respond to GABA concentrations similar to $GABA_ARs$ expressed in the brain. Comparable effects of GABA and muscimol have been reported in activated human peripheral lymphocytes where both have elicited macroscopic currents.[22] In whole cell clamp studies, tonic currents were observed in GABA activated encephalitogenic T lymphocytes.[21] Importantly, we showed for the first time that $CD4^+$ T cell responded directly to selective $GABA_AR$ modulators in the presence of GABA with a change of current response. The lung eosinophilic inflammatory response to ovalbumin allergen was attenuated with orally administered positive allosteric $GABA_AR$ modulators. Further investigation of different leukocyte populations demonstrated that at least compound **1** affected the number of $CD4^+$ T cells in BALF and changed concentration-dependently their transmembrane currents. The effect on T cells was not significant for $\alpha_5\beta_3\gamma_2$ selective $GABA_AR$ ligand compound **2** as reflected in both the $CD4^+$ T cell population in BALF and change of current response *ex vivo*. Evidence for $GABA_AR$ subunit expression for this cell type include reports of splenic $CD4^+$ T cells obtained from NOD mice that expressed the α_1 subunits but not the α_5 subunits.[24] In addition, encephalitogenic T cells from mice have been reported to express the α_1, α_4, β_3, and δ subunits under resting and activated conditions.[21] Still, the exact mechanism by which changes in T cell transmembrane currents reduce inflammation remains to be elucidated.

In summary, we have demonstrated that splenocytes express functional $GABA_ARs$ that can be modulated with small molecules. In addition, oral administration of compounds **1** and **2** relaxed guinea pig airway smooth muscle *ex vivo* and alleviated AHR in murine model of asthma without increasing mucous metaplasia. Thus, we demonstrated for the first time a new oral asthma drug strategy based on effective prototype ligands that can be optimized further to combat both AHR and airway inflammation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.7b00183.

Supplemental Experimental Procedures and 12 figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: arnold2@uwm.edu.

ORCID

Michael Rajesh Stephen: 0000-0003-0027-6560

James M. Cook: 0000-0001-5512-3022

Leggy A. Arnold: 0000-0003-1411-1572

Funding

This work was supported by the University of Wisconsin-Milwaukee, the National Institutes of Health R03DA031090 (L.A.A.), R01NS076517 (J.M.C., L.A.A.), R01HL118561 (J.M.C., L.A.A., C.W.E., D.C.S.), R01MH096463 (J.M.C.,.A.A.), R01GM065281 (C.W.E., G.T.Y., J.M.C.), and R01HL122340 (C.W.E.), as well as the University of Wisconsin Milwaukee Research Foundation (Catalyst grant), the Lynde and Harry Bradley Foundation, the Richard and Ethel Herzfeld Foundation, the Stony Wold-Herbert Fund (G.T.Y.), and the Foundation for Anesthesia Education and Research (G.T.Y.).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Beryl R. Forman and Jennifer L. Nemke (Animal Facility at UWM) for their guidance and support.

■ ABBREVIATIONS

$GABA_AR$, $GABA_A$ receptor; AHR, airway hyperresponsiveness; CNS, central nervous system; PMA, phorbol myristate acetate; PHA, phytohemagglutinin; NMR, nuclear magnetic resonance; HRMS, high resolution mass spectrometry; BBB, blood−brain barrier; ECS, external cell solution; ICS, internal cell solution; DMSO, dimethyl sulfoxide; BALF, bronchoalveolar lavage fluid; GABA, gamma aminobutyric acid; sRaw, specific airway resistance; β_2AR, beta 2 adrenergic receptor

■ REFERENCES

(1) Trends in Asthma Morbidity and Mortality. American Lung Association Epidemiology & Statistics Unit Research and Program Services.

(2) National Heart, Lung, and Blood Institute. Guidelines for the Diagnosis and Management of Asthma, Full Report (August 2007).
(3) Pascual, R. M.; Peters, S. P. Airway remodeling contributes to the progressive loss of lung function in asthma: an overview. *J. Allergy Clin. Immunol.* **2005**, *116*, 477−486 quiz 487.
(4) Everard, M. L. Aerosol therapy past, present, and future: a clinician's perspective. *Respir Care* **2000**, *45*, 769−776.
(5) Cates, C. J.; Cates, M. J. Regular treatment with salmeterol for chronic asthma: serious adverse events. *Cochrane Database Syst. Rev.* **2008**, CD006363.
(6) Cates, C. J.; Cates, M. J. Regular treatment with formoterol for chronic asthma: serious adverse events. *Cochrane Database Syst. Rev.* **2012**, CD006923.
(7) Dahl, R. Systemic side effects of inhaled corticosteroids in patients with asthma. *Respir Med.* **2006**, *100*, 1307−1317.
(8) Kelly, H. W.; Sternberg, A. L.; Lescher, R.; Fuhlbrigge, A. L.; Williams, P.; Zeiger, R. S.; Raissy, H. H.; Van Natta, M. L.; Tonascia, J.; Strunk, R. C.; Group, C. R. Effect of inhaled glucocorticoids in childhood on adult height. *N. Engl. J. Med.* **2012**, *367*, 904−912.
(9) Lipworth, B. J. Systemic adverse effects of inhaled corticosteroid therapy: A systematic review and meta-analysis. *Arch. Intern. Med.* **1999**, *159*, 941−955.
(10) Zhang, L.; Prietsch, S. O.; Ducharme, F. M. Inhaled corticosteroids in children with persistent asthma: effects on growth. *Cochrane Database Syst. Rev.* **2014**, CD009471.
(11) Montuschi, P.; Peters-Golden, M. L. Leukotriene modifiers for asthma treatment. *Clin. Exp. Allergy* **2010**, *40*, 1732−1741.
(12) Lima, J. J.; Zhang, S.; Grant, A.; Shao, L. H.; Tantisira, K. G.; Allayee, H.; Wang, L. W.; Sylvester, J.; Holbrook, J.; Wise, R.; Weiss, S. T.; Barnes, K. Influence of leukotriene pathway polymorphisms on response to montelukast in asthma. *Am. J. Respir. Crit. Care Med.* **2006**, *173*, 379−385.
(13) Mortensen, M.; Patel, B.; Smart, T. G. GABA Potency at GABA(A) Receptors Found in Synaptic and Extrasynaptic Zones. *Front. Cell. Neurosci.* **2011**, *6*, 1.
(14) Olsen, R. W.; Sieghart, W. International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev.* **2008**, *60*, 243−260.
(15) Clayton, T.; Poe, M. M.; Rallapalli, S.; Biawat, P.; Savic, M. M.; Rowlett, J. K.; Gallos, G.; Emala, C. W.; Kaczorowski, C. C.; Stafford, D. C.; Arnold, L. A.; Cook, J. M. A Review of the Updated Pharmacophore for the Alpha 5 GABA(A) Benzodiazepine Receptor Model. *Int. J. Med. Chem.* **2015**, *2015*, 430248.
(16) Gallos, G.; Yim, P.; Chang, S.; Zhang, Y.; Xu, D.; Cook, J. M.; Gerthoffer, W. T.; Emala, C. W., Sr. Targeting the restricted alpha-subunit repertoire of airway smooth muscle GABAA receptors augments airway smooth muscle relaxation. *Am. J. Physiol Lung Cell Mol. Physiol* **2012**, *302*, L248−256.
(17) Gallos, G.; Yocum, G. T.; Siviski, M. E.; Yim, P. D.; Fu, X. W.; Poe, M. M.; Cook, J. M.; Harrison, N.; Perez-Zoghbi, J.; Emala, C. W., Sr. Selective targeting of the alpha5-subunit of GABAA receptors relaxes airway smooth muscle and inhibits cellular calcium handling. *Am. J. Physiol Lung Cell Mol. Physiol* **2015**, *308*, L931−942.
(18) Mizuta, K.; Xu, D.; Pan, Y.; Comas, G.; Sonett, J. R.; Zhang, Y.; Panettieri, R. A., Jr.; Yang, J.; Emala, C. W., Sr. GABAA receptors are expressed and facilitate relaxation in airway smooth muscle. *Am. J. Physiol Lung Cell Mol. Physiol* **2008**, *294*, L1206−1216.
(19) Xiang, Y. Y.; Wang, S.; Liu, M.; Hirota, J. A.; Li, J.; Ju, W.; Fan, Y.; Kelly, M. M.; Ye, B.; Orser, B.; O'Byrne, P. M.; Inman, M. D.; Yang, X.; Lu, W. Y. A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. *Nat. Med.* **2007**, *13*, 862−867.
(20) Bhat, R.; Axtell, R.; Mitra, A.; Miranda, M.; Lock, C.; Tsien, R. W.; Steinman, L. Inhibitory role for GABA in autoimmune inflammation. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 2580−2585.
(21) Bjurstom, H.; Wang, J.; Ericsson, I.; Bengtsson, M.; Liu, Y.; Kumar-Mendu, S.; Issazadeh-Navikas, S.; Birnir, B. GABA, a natural immunomodulator of T lymphocytes. *J. Neuroimmunol.* **2008**, *205*, 44−50.
(22) Dionisio, L.; De Rosa, M. J.; Bouzat, C.; Esandi, M. d. C. An intrinsic GABAergic system in human lymphocytes. *Neuropharmacology* **2011**, *60*, 513−519.
(23) Forkuo, G. S.; Guthrie, M. L.; Yuan, N. Y.; Nieman, A. N.; Kodali, R.; Jahan, R.; Stephen, M. R.; Yocum, G. T.; Treven, M.; Poe, M. M.; Li, G.; Yu, O. B.; Hartzler, B. D.; Zahn, N. M.; Ernst, M.; Emala, C. W.; Stafford, D. C.; Cook, J. M.; Arnold, L. A. Development of GABAA Receptor Subtype-Selective Imidazobenzodiazepines as Novel Asthma Treatments. *Mol. Pharmaceutics* **2016**, *13*, 2026−2038.
(24) Tian, J.; Lu, Y.; Zhang, H.; Chau, C. H.; Dang, H. N.; Kaufman, D. L. Gamma-aminobutyric acid inhibits T cell autoimmunity and the development of inflammatory responses in a mouse type 1 diabetes model. *J. Immunol.* **2004**, *173*, 5298−5304.
(25) Munroe, M. E.; Businga, T. R.; Kline, J. N.; Bishop, G. A. Anti-inflammatory effects of the neurotransmitter agonist Honokiol in a mouse model of allergic asthma. *J. Immunol.* **2010**, *185*, 5586−5597.
(26) Ai, J.; Wang, X.; Nielsen, M. Honokiol and magnolol selectively interact with GABAA receptor subtypes in vitro. *Pharmacology* **2001**, *63*, 34−41.
(27) Yocum, G. T.; Gallos, G.; Zhang, Y.; Jahan, R.; Stephen, M. R.; Varagic, Z.; Puthenkalam, R.; Ernst, M.; Cook, J. M.; Emala, C. W. Targeting the gamma-Aminobutyric Acid A Receptor alpha4 Subunit in Airway Smooth Muscle to Alleviate Bronchoconstriction. *Am. J. Respir. Cell Mol. Biol.* **2016**, *54*, 546−553.
(28) Stamenic, T. T.; Poe, M. M.; Rehman, S.; Santrac, A.; Divovic, B.; Scholze, P.; Ernst, M.; Cook, J. M.; Savic, M. M. Ester to amide substitution improves selectivity, efficacy and kinetic behavior of a benzodiazepine positive modulator of GABAA receptors containing the alpha5 subunit. *Eur. J. Pharmacol.* **2016**, *791*, 433−443.
(29) Akinci, M. K.; Schofield, P. R. Widespread expression of GABA(A) receptor subunits in peripheral tissues. *Neurosci. Res.* **1999**, *35*, 145−153.
(30) Tyagi, N.; Lominadze, D.; Gillespie, W.; Moshal, K. S.; Sen, U.; Rosenberger, D. S.; Steed, M.; Tyagi, S. C. Differential expression of gamma-aminobutyric acid receptor A (GABA(A)) and effects of homocysteine. *Clin. Chem. Lab. Med.* **2007**, *45*, 1777−1784.
(31) Shen, Y.; Lindemeyer, A. K.; Spigelman, I.; Sieghart, W.; Olsen, R. W.; Liang, J. Plasticity of GABAA receptors after ethanol pre-exposure in cultured hippocampal neurons. *Mol. Pharmacol.* **2011**, *79*, 432−442.
(32) Garcia-Recio, S.; Gascon, P. Biological and Pharmacological Aspects of the NK1-Receptor. *BioMed Res. Int.* **2015**, *2015*, 495704.
(33) Roffel, A. F.; Elzinga, C. R.; Zaagsma, J. Muscarinic M3 receptors mediate contraction of human central and peripheral airway smooth muscle. *Pulm. Pharmacol.* **1990**, *3*, 47−51.
(34) Puthenkalam, R.; Treven, M.; Ramerstorfer, J.; Steudle, F.; Scholze, P.; Gallos, G.; Poe, M. M.; Methuku, K. R.; Li, G.; Arnold, L. A.; Sieghart, W.; Santrac, A.; Savic, M. M.; Emala, C. W.; Cook, J. M.; Ernst, M. Benzodiazepine ligands with improved α5-selectivity and their airway smooth muscle relaxant effect. *Mol. Pharmacol.* **2016**, submitted.
(35) Henderson, W. R., Jr.; Lewis, D. B.; Albert, R. K.; Zhang, Y.; Lamm, W. J.; Chiang, G. K.; Jones, F.; Eriksen, P.; Tien, Y. T.; Jonas, M.; Chi, E. Y. The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J. Exp. Med.* **1996**, *184*, 1483−1494.
(36) Lee, J. J.; McGarry, M. P.; Farmer, S. C.; Denzler, K. L.; Larson, K. A.; Carrigan, P. E.; Brenneise, I. E.; Horton, M. A.; Haczku, A.; Gelfand, E. W.; Leikauf, G. D.; Lee, N. A. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J. Exp. Med.* **1997**, *185*, 2143−2156.
(37) Chauhan, B. F.; Ducharme, F. M. Addition to inhaled corticosteroids of long-acting beta2-agonists versus anti-leukotrienes for chronic asthma. *Cochrane Database Syst. Rev.* **2014**, CD003137.
(38) Nelson, H. S.; Nathan, R. A.; Kalberg, C.; Yancey, S. W.; Rickard, K. A. Comparison of inhaled salmeterol and oral zafirlukast in asthmatic patients using concomitant inhaled corticosteroids. *MedGenMed.* **2001**, *3*, 3.

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

(39) Cohn, R. C. A review of the effects of medication delivery systems on treatment adherence in children with asthma. *Curr. Ther. Res.* **2003**, *64*, 34−44.
(40) Fabbri, L. M.; Piattella, M.; Caramori, G.; Ciaccia, A. Oral vs inhaled asthma therapy. *Drugs* **1996**, *52* (Suppl 6), 20−28.
(41) Jahan, R.; Stephen, M. R.; Forkuo, G. S.; Kodali, R.; Guthrie, M. L.; Nieman, A. N.; Yuan, N.; Zahn, N. M.; Poe, M. M.; Li, G.; Yu, O. B.; Yocum, G. T.; Emala, C. W.; Stafford, D. C.; Cook, J. M.; Arnold, L. A. Optimization of Substituted Imidazobenzodiazepines as Novel Asthma Treatments. *Eur. J. Med. Chem.* **2016**, *126*, 550−560.
(42) Gosens, R.; Zaagsma, J.; Meurs, H.; Halayko, A. J. Muscarinic receptor signaling in the pathophysiology of asthma and COPD. *Respir. Res.* **2006**, *7*, 73.
(43) Guilbert, T. W.; Morgan, W. J.; Zeiger, R. S.; Mauger, D. T.; Boehmer, S. J.; Szefler, S. J.; Bacharier, L. B.; Lemanske, R. F., Jr.; Strunk, R. C.; Allen, D. B.; Bloomberg, G. R.; Heldt, G.; Krawiec, M.; Larsen, G.; Liu, A. H.; Chinchilli, V. M.; Sorkness, C. A.; Taussig, L. M.; Martinez, F. D. Long-term inhaled corticosteroids in preschool children at high risk for asthma. *N. Engl. J. Med.* **2006**, *354*, 1985−1997.
(44) Sholter, D. E.; Armstrong, P. W. Adverse effects of corticosteroids on the cardiovascular system. *Can. J. Cardiol* **2000**, *16*, 505−511.
(45) Ciriaco, M.; Ventrice, P.; Russo, G.; Scicchitano, M.; Mazzitello, G.; Scicchitano, F.; Russo, E. Corticosteroid-related central nervous system side effects. *J. Pharmacol. Pharmacother.* **2013**, *4*, S94−98.
(46) Cook, J. M., Clayton, T., Hiteshkumar, D., Rallapalli, S., Johnson, Y. T., Yang, J., Poe, M. M., Namjoshi, O. A., Wang, Z.-J. GABAergic receptor subtype selective ligands and their uses (2012). US 20150258128.
(47) Forkuo, G. S.; Kim, H.; Thanawala, V. J.; Al-Sawalha, N.; Valdez, D.; Joshi, R.; Parra, S.; Pera, T.; Gonnella, P. A.; Knoll, B. J.; Walker, J. K.; Penn, R. B.; Bond, R. A. PDE4 Inhibitors Attenuate the Asthma Phenotype Produced by beta2-adrenoceptor Agonists in PNMT-KO Mice. *Am. J. Respir. Cell Mol. Biol.* **2016**, *55*, 234.
(48) Glaab, T.; Taube, C.; Braun, A.; Mitzner, W. Invasive and noninvasive methods for studying pulmonary function in mice. *Respir. Res.* **2007**, *8*, 63.
(49) van Rijt, L. S.; Kuipers, H.; Vos, N.; Hijdra, D.; Hoogsteden, H. C.; Lambrecht, B. N. A rapid flow cytometric method for determining the cellular composition of bronchoalveolar lavage fluid cells in mouse models of asthma. *J. Immunol. Methods* **2004**, *288*, 111−121.

DOI: 10.1021/acs.molpharmaceut.7b00183
Mol. Pharmaceutics XXXX, XXX, XXX−XXX

Supporting Information

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

Gloria S. Forkuo,† Amanda N. Nieman,† Nina Y. Yuan,† Revathi Kodali,† Ted W. Harris,† Olivia B. Yu,† Nicolas M. Zahn,† Rajwana Jahan,† Guanguan Li,† Michael Rajesh Stephen,† Margaret L. Guthrie,† Michael M. Poe,† Benjamin D. Hartzler,† Gene T. Yocum,‡ Charles W. Emala,‡ Douglas A. Steeber,§ Douglas C. Stafford,† James M. Cook,† and Leggy A. Arnold †,*

†Department of Chemistry and Biochemistry and the Milwaukee Institute for Drug Discovery, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, 53201.
‡Department of Anesthesiology, Columbia University, New York, New York, 10032.
§Department of Biological Sciences, University of Wisconsin-Milwaukee, Milwaukee, Wisconsin, 53201.

Figure S1 (related to Figure 5). 2
Figure S2 (related to Figure 5). 2
Figure S3 (related to Figure 6B, where quantification is displayed). 3
Figure S4 (related to Figure 6). 3
Figure S5 (related to Figure 6). 4
Figure S6 (related to Figure 6). 4
Figure S7 (related to Figure 6). 5
Figure S8 (related to Figure 6). 5
Figure S9 (related to Figure 1). 6
Figure S10 (related to Figure 1). 6
Figure S11 (related to Figure 1). 7
Figure S12 (related to Figure 1). 7
Figure S13 (related to Figure 1). 8
Figure S14 (related to Figure 1). 8
Supplemental experimental procedures 9
Airway Smooth Muscle relaxation. 9
Rotarod assay: 9
Patch clamp assay: 10
Histopathological analysis of lung sections: 10
Mice Pharmacokinetic study: 10
Reference 11

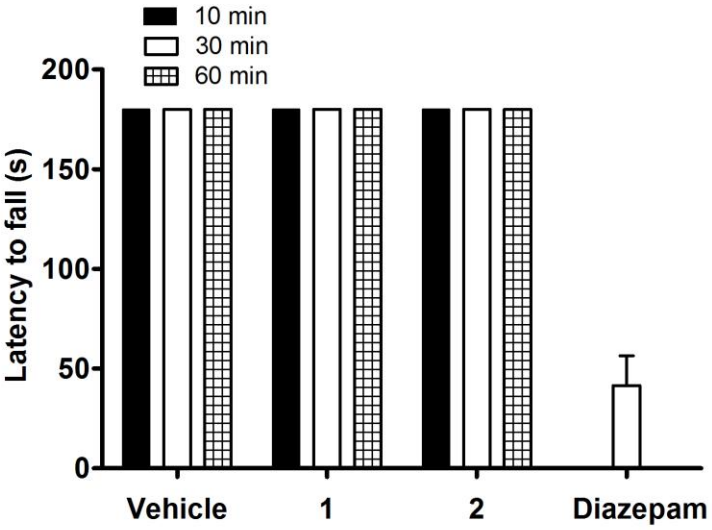


Figure S1 (related to Figure 5). Effect of 1 and 2 on sensorimotor coordination. Swiss Webster mice received a single intra-gastric gavage of test compound (100 mg/kg) or diazepam (5 mg/kg ip) and placed on a rotarod at 15rpm for 3 minutes after 10, 30 and 60 minutes of drug administration. A fail was 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.

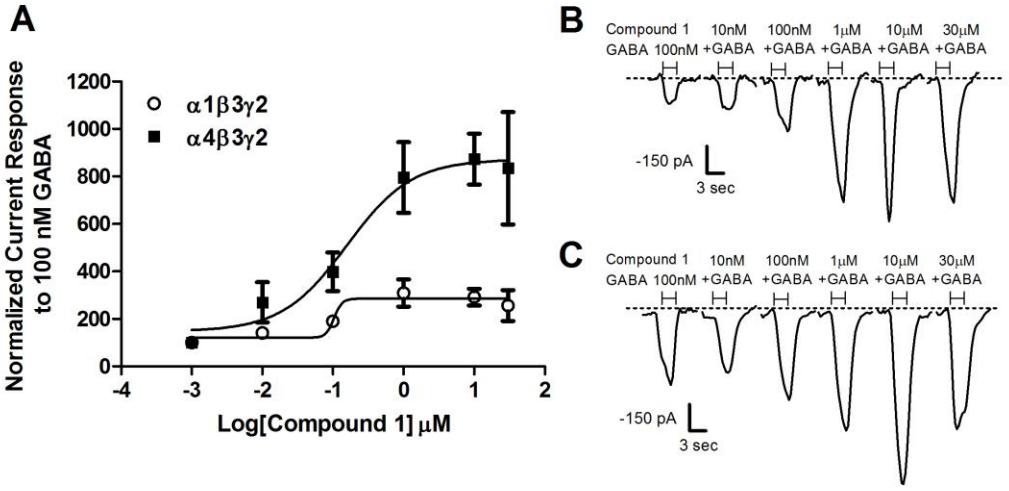


Figure S2 (related to Figure 5). Automated patch clamp with compound 1. A) Concentration-dependent negative current responses in the presence of EC_{20} concentration of GABA and increasing concentration of compound 1 applied together for 3 seconds using α1β3γ2 or α4β3γ2 $GABA_AR$ expressing HEK293T 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.

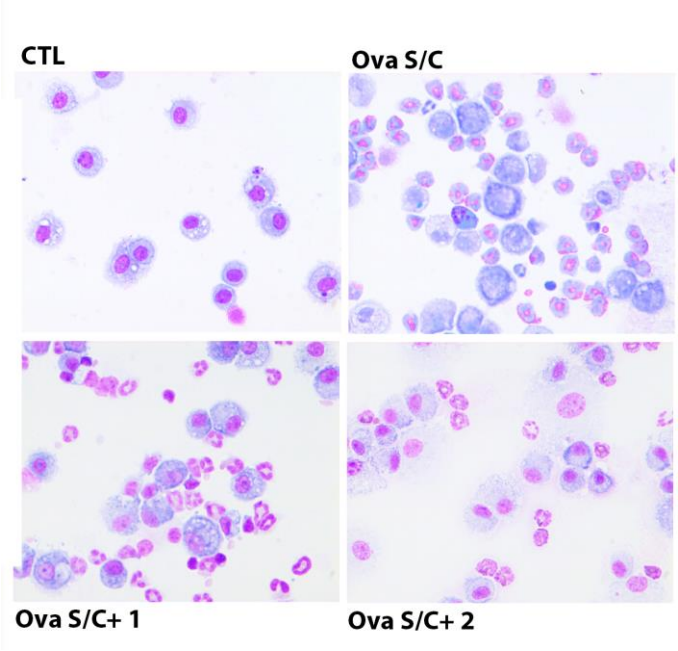


Figure S3 (related to Figure 6B, where quantification is displayed).
Effect of 1 and 2 on airway eosinophilia in BALF.
Representative images of Wright Giemsa stained slides. Ova s/c BALB/c mice were administered 1 or 2 via oral gavage, 100 mg/kg twice daily for 5 days. Data represent % normalized eosinophils 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.

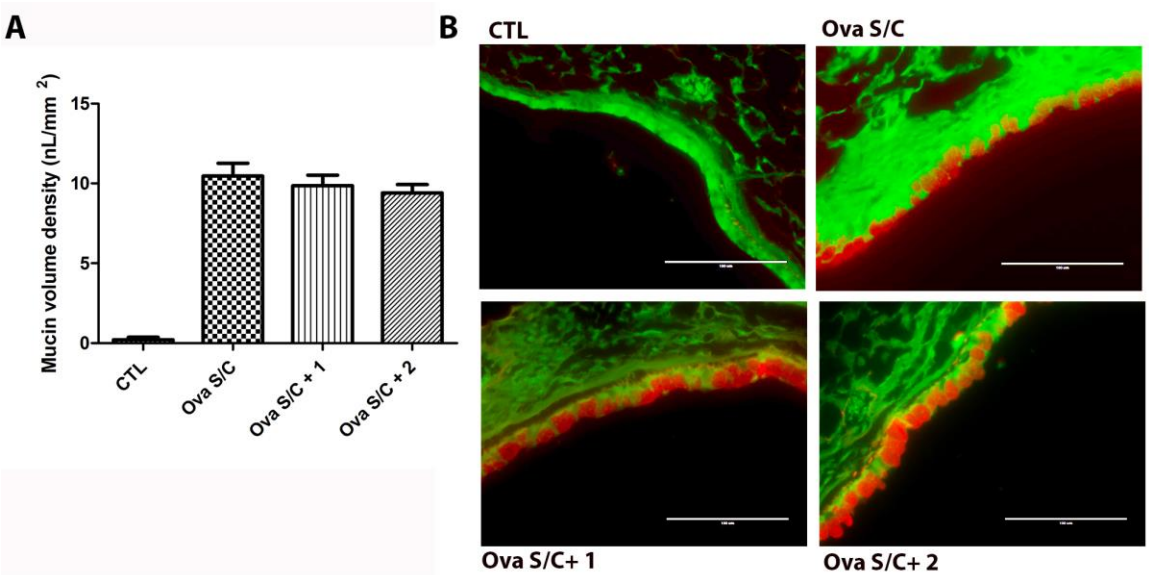


Figure S4 (related to Figure 6). Effect of 1 and 2 on mucous production
Morphometric quantification of mucin volume density and B) representative images of mucin (red) in the airway epithelium (green) with periodic acid fluorescent Schiff's stain. Ova s/c BALB/c mice were administered 1 via oral gavage, 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.

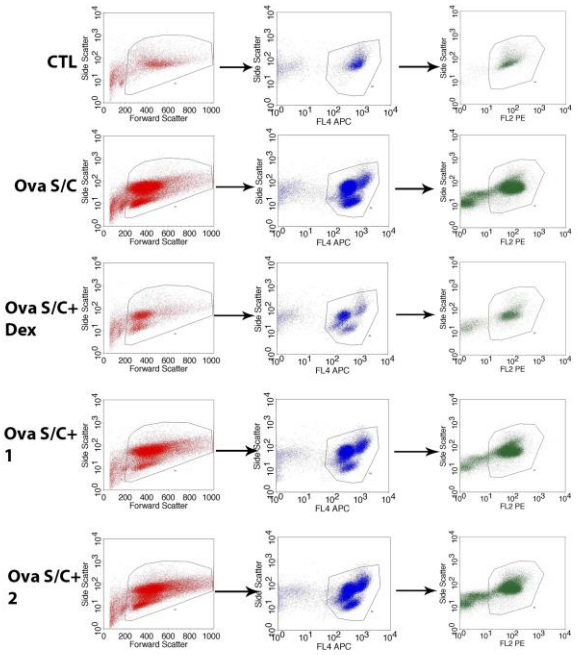


Figure S5 (related to Figure 6). Gating strategy for CCR3+ cells in BALF of ova s/c BALB/c mice administered dexamethasone i.p., 4 mg/kg daily for 8 days; 1 via oral gavage, 100 mg/kg twice daily for 5 days and 2 via oral gavage, 100 mg/kg twice daily for 5 days.

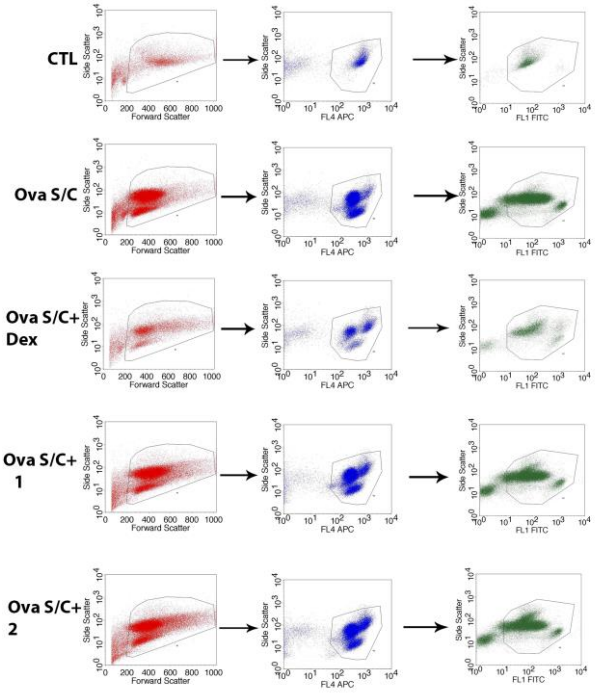


Figure S6 (related to Figure 6). Gating strategy for GR1+ cells in BALF of ova s/c BALB/c mice administered dexamethasone i.p., 4 mg/kg daily for 8 days; 1 via oral gavage, 100 mg/kg twice daily for 5 days and 2 via oral gavage, 100 mg/kg twice daily for 5 days.

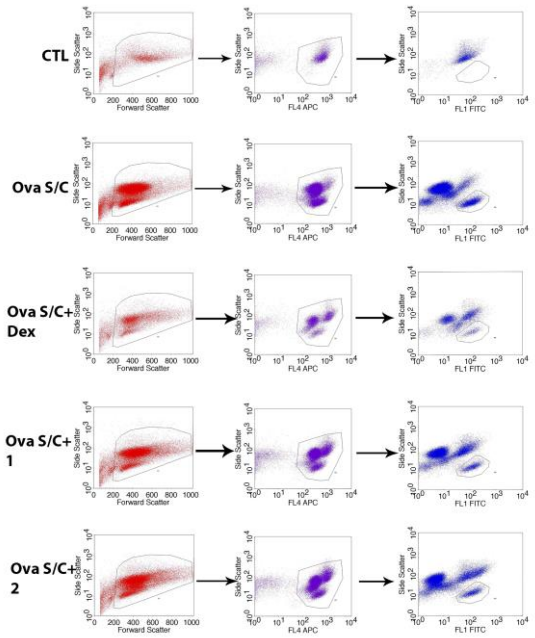


Figure S7 (related to Figure 6). Gating strategy for CD4+ cells in BALF of ova s/c BALB/c mice administered dexamethasone i.p., 4 mg/kg daily for 8 days; 1 via oral gavage, 100 mg/kg twice daily for 5 days and 2 via oral gavage, 100 mg/kg twice daily for 5 days.

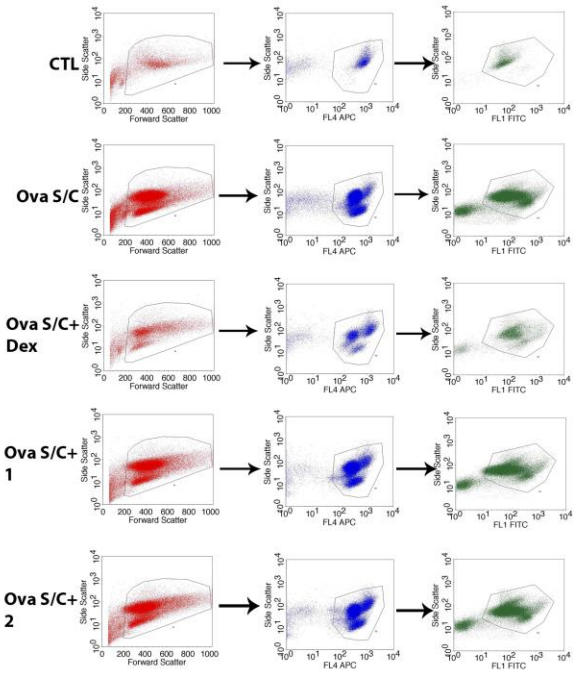


Figure S8 (related to Figure 6). Gating strategy for CD11b+ cells in BALF of ova s/c BALB/c mice administered dexamethasone i.p., 4 mg/kg daily for 8 days; 1 via oral gavage, 100 mg/kg twice daily for 5 days and 2 via oral gavage, 100 mg/kg twice daily for 5 days.

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.

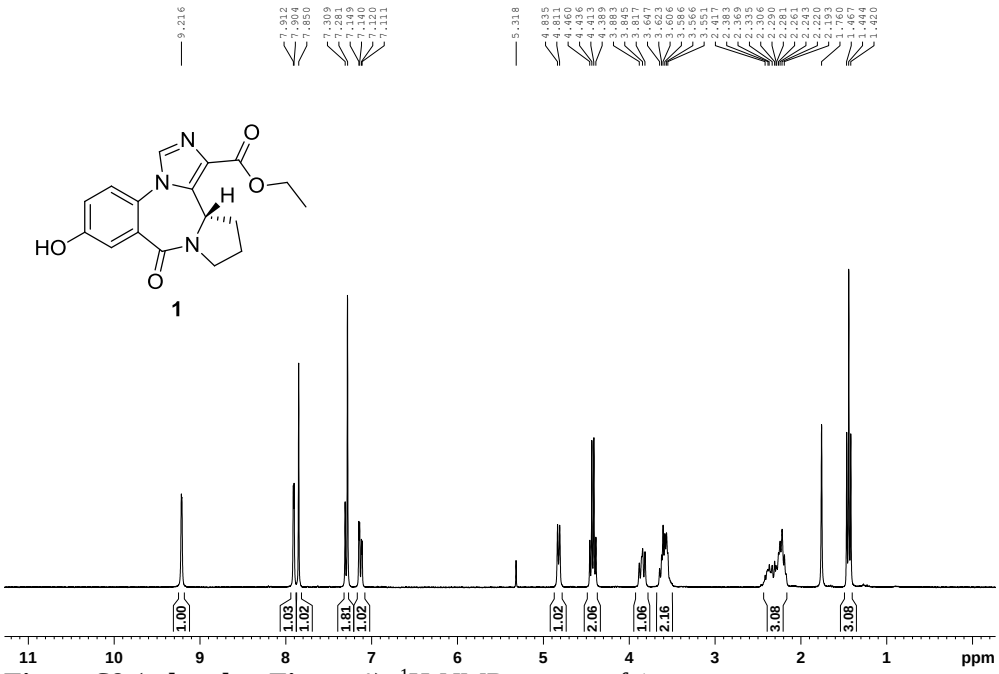


Figure S9 (related to Figure 1). ^{1}H-NMR spectra of 1.

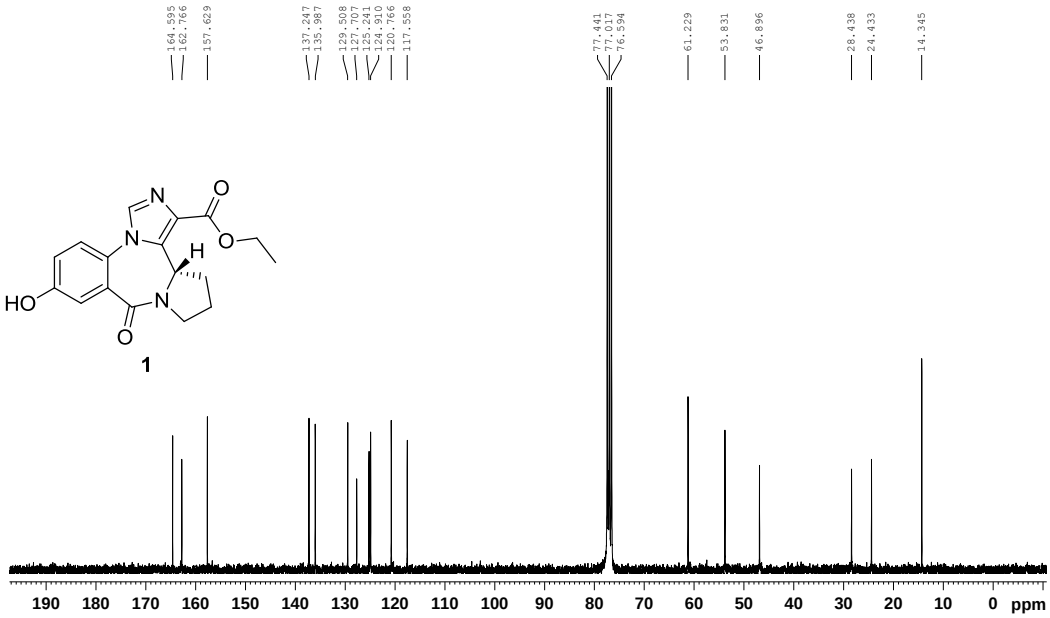


Figure S10 (related to Figure 1). ^{13}C-NMR spectra of 1.

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

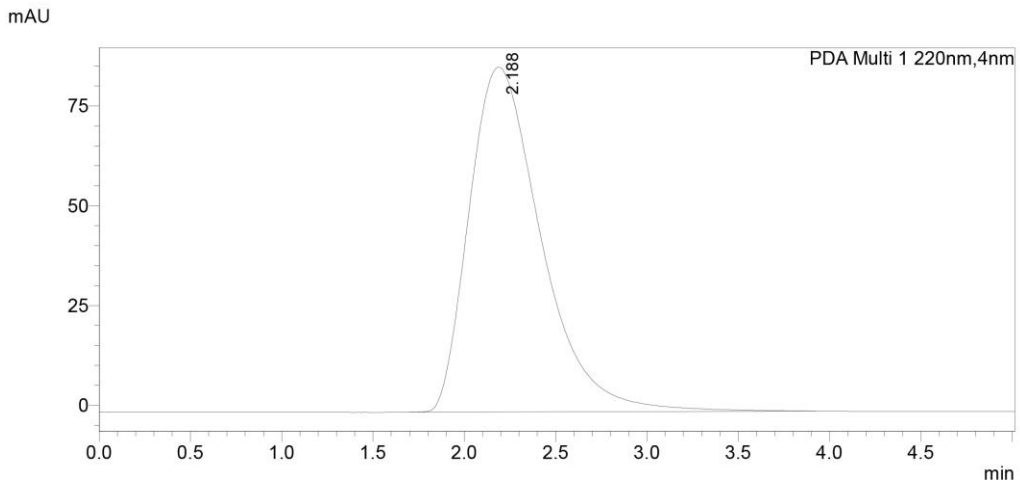


Figure S11 (related to Figure 1). HLPC run of 1.

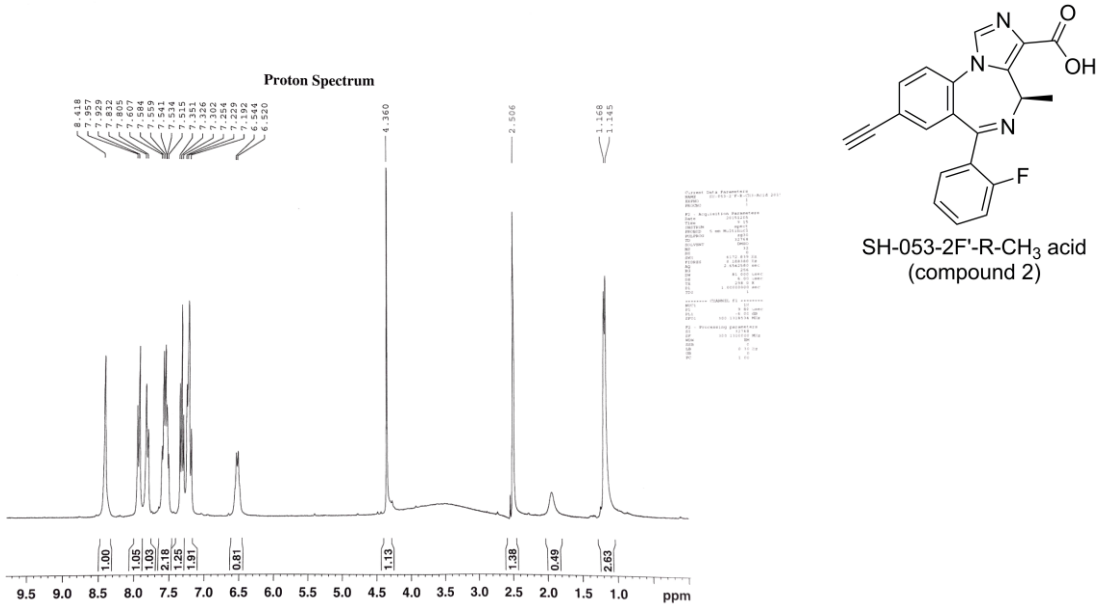


Figure S12 (related to Figure 1). ^{1}H-NMR spectra of 2.

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.

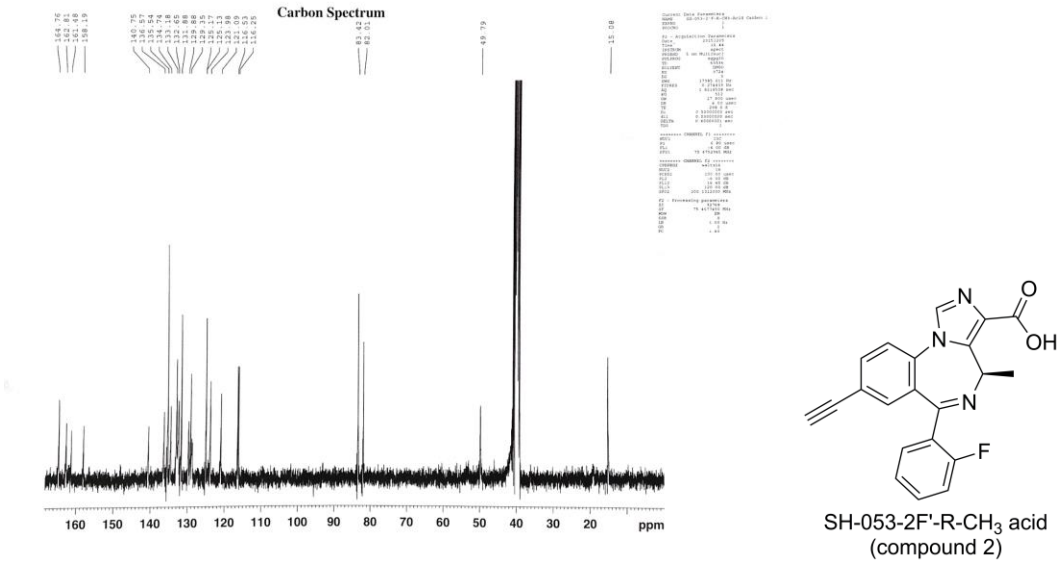


Figure S13 (related to Figure 1). ^{13}C-NMR spectra of 2.

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

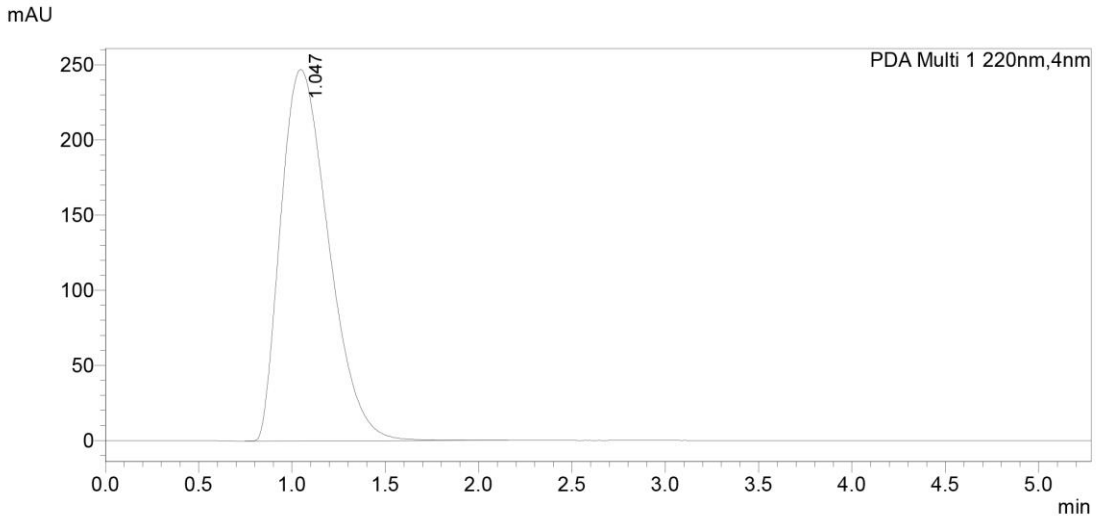


Figure S14 (related to Figure 1). HPLC run of 2.

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.
Forkuo, G.S., Guthrie, M.L., Yuan, N.Y., Nieman, A.N., Kodali, R., Jahan, R., Stephen, M.R., Yocum, G.T., Treven, M., Poe, M.M., *et al.* (2016). Development of GABAA Receptor Subtype-Selective Imidazobenzodiazepines as Novel Asthma Treatments. Molecular pharmaceutics *13*, 2026-2038.

Piccotti, L., Dickey, B.F., and Evans, C.M. (2012). Assessment of intracellular mucin content in vivo. Methods Mol Biol *842*, 279-295.