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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Abstract

We describe lead compound MIDD0301 for the oral treatment of asthma based on previously developed positive allosteric $\alpha_5\beta_3\gamma_2$ selective $GABA_A$ receptor ($GABA_AR$) modulators. MIDD0301 relaxed airway smooth muscle at single micromolar concentrations as demonstrated with *ex vivo* guinea pig tracheal rings. MIDD0301 also attenuated airway hyperresponsiveness (AHR) in an ovalbumin murine model of asthma by oral administration. Reduced numbers of eosinophils and macrophages were observed in mouse broncho-alveolar lavage fluid without changing mucous metaplasia. Importantly, lung cytokine expression of IL-17A, IL-4, and TNF-α were reduced for MIDD0301 treated mice without changing anti-inflammatory cytokine IL-10 levels. Automated patch clamp confirmed amplification of GABA induced current mediated by $\alpha_{1\text{-}3,5}\beta_3\gamma_2$ $GABA_ARs$ in the presence of MIDD0301. Pharmacodynamically, transmembrane currents of *ex vivo* $CD4^+$ T cells from asthmatic mice were modulated by MIDD0301 in the presence of GABA. The number of $CD4^+$ T cell observed in the lung of MIDD0301 treated mice were reduced by an oral treatment of 20 mg/kg b.i.d. for 5 days. A half-life of almost 14 hours was demonstrated by pharmacokinetic studies (PK) with no adverse CNS effects when treated mice were subjected to sensorimotor studies using the rotarod. PK studies also confirmed very low brain distribution. In conclusion, MIDD0301 represents a safe and improved oral asthma drug candidate that relaxes airway smooth muscle and attenuates inflammation in the lung leading to a reduction of AHR at a dosage lower than earlier reported $GABA_AR$ ligands.

INTRODUCTION

Asthma is characterized by chronic inflammation of the airways resulting in hyperactivity to external stimuli and airway obstruction.[1] Common clinical features include recurrence or

episodes of cough, chest tightness, shortness of breath, and wheezing. Asthma is a major global health concern, estimated at 300 million people affected worldwide, with 21 million in the United States alone. Global asthma prevalence is estimated to reach 400 million in year 2025.[2, 3] Despite the growing challenge of asthma, currently approved therapeutic options are limited.

The preferred therapy for chronic persistent asthma includes inhaled corticosteroids and inhaled long acting β2 adrenergic receptor agonists (LABAs).[4] Aerosol administration allows for targeted lung drug delivery, thus avoiding systemic side effects associated with these drugs. Nevertheless, asthma morbidity and mortality continues to rise. One important factor is the failure of patients to properly use inhaled medications leading to non-compliance and loss of asthma control.[5, 6] An alternative for patients whose symptoms are poorly controlled with inhaled steroids is oral leukotriene receptor antagonists.[2] However, genetic variations in leukotriene signaling genes may preclude their widespread efficacy.[7] It has been reported that 35-78% of patients are nonresponsive to such medications.[8, 9] Finally, injectable biologics such as Omalizumab and Meolizumab were developed to reduce asthma lung inflammation, but patient costs for these drugs of up to $30,000 a year limits their use to only severe disease.[10, 11] These biologics are also unable to protect against acute bronchospasm and present anaphylaxis risks.

An improved drug for asthma would alleviate the multiple acute and chronic disease symptoms such as airway smooth muscle constriction and airway inflammation. Importantly, it should be orally active to eliminate poor aerosol compliance and have limited cardiovascular and central nervous system (CNS) adverse effects. To address these needs, our laboratory has advanced the development of a new asthma medication based on positive allosteric subtype selective $GABA_A$ receptor modulation. These compounds do not cross the blood brain barrier and target asthma pathophysiology without systemic side effects.[12-14] The $GABA_A$ receptor ($GABA_AR$) is a

ligand-gated chloride ion channel best known for its role in CNS inhibitory neurotransmission. $GABA_AR$s are heteropentameric membrane receptors mainly consisting of combinations of 19 different subunits ($\alpha_{1–6}$, $\beta_{1–3}$, $\gamma_{1–3}$, δ, ε, π, θ, ρ_{1-3}). Classical $GABA_AR$s consist of 2 alpha, 2 beta, and one "tertiary" subunit (γ, δ, ε, θ, or π).[15, 16] Importantly, discrete $GABA_AR$ subtypes have been identified in airway smooth muscle, airway epithelium, and inflammatory cells.[12, 13, 17-21] Their activation by positive allosteric modulators relaxed isolated pre-constricted airway smooth muscles and reduced inflammation and airway hyperresponsiveness (AHR) in murine asthma models.[12, 13, 18, 19, 21-25] Notably, an $\alpha_5\beta_3\gamma_2$ $GABA_AR$ selective compound 2 (Figure 1) attenuated asthma disease measures in ovalbumin sensitized and challenged (ova s/c) BALB/c mice at a dose of 100 mg/kg for 5 days.[13]

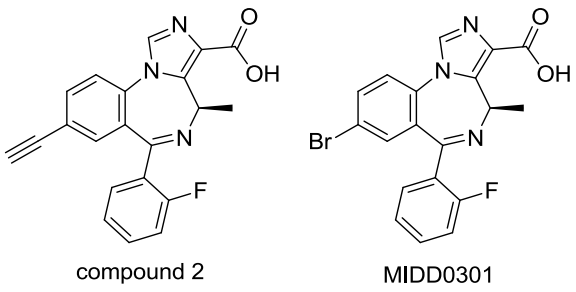


Figure 1. Structures of $\alpha_5\beta_3\gamma_2$ $GABA_AR$ selective positive allosteric modulators

In addition, the number of leukocytes, especially eosinophils, in ova s/c mouse lungs were reduced with compound 2 treatment. However, the number of lung $CD4^+$ T cells was not reduced statistically compared to vehicle-treated mice, and electrophysiological analysis showed that compound 2 modulation of GABA-induced current responses of isolated $CD4^+$ T cells from ova s/c mice was negligible. To improve the performance of compound 2, further development

produced lead compound MIDD0301 (Figure 1) that reduced AHR at lower dosage, significantly limited the number of $CD4^+$ T cells, and reduced the expression of pro-inflammatory cytokines.

EXPERIMENTAL SECTION

Chemicals. MIDD0301 was synthesized using a published procedure.[26] The purity was of >98% was confirmed by HPLC. Identity was determined by ^{1}H-NMR, ^{13}C-NMR, and high resolution mass spectroscopy. (Supporting Information for spectra)

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 groups 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.[27] Control mice were sensitized with ova and challenged with i.n. saline. The effects of MIDD0301 at 20, 50 and 100 mg/kg administered 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 ova s/c BALB/c mice received salmeterol at 1 mg/kg b.i.d. for 5 days. Airway hyper-responsiveness 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.

Drug treatment protocol. Sterile solutions of MID0301 and salmeterol 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. MIDD0301 or salmeterol were administered by oral gavage at different concentrations 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 compound just before airway parameter measurements. Mice were monitored daily after drug administration.

Airway smooth muscle relaxation. Guinea pig experiments were approved by 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 were 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 MDD0301, 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.

Assessment of airway hyperresponsiveness. AHR to methacholine in conscious, spontaneously breathing animals was measured by DSI's Buxco FinePointe Non-Invasive Airway Mechanics (NAM) instrument [28]. 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[12, 13].

Drug pharmacokinetic studies, rotarod studies and patch clamp studies with transient transfected cells. (see Supporting Information)

Automated patch-clamp with $CD4^+$ T-lymphocytes. Four BALB/c mouse spleens were isolated and excised through a strainer using the plunger of a syringe. The cells were washed through the strainer with PBS and centrifuged for 5 min at 1,600 rpm. After aspiration of the supernatant the cell pellet was resuspended in BD Pharm Lyse lysing solution (BD Biosciences, San Jose, CA), followed by incubation (2 min at 37°C), addition of 30 ml PBS and centrifugation (5 minutes at 1,600 rpm). $CD4^+$ 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). After isolation, cells were centrifuged at 380g for 2 min and gently suspended in extracellular solution (in mM: NaCl 140, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 10, pH 7.4 with NaOH) at a concentration of $5x10^6$ cells/ml. Automated patch clamp assays were conducted with the IonFlux16 as described previously.[12, 13] Briefly, the IonFlux16 plates consist of 8 patterns, each containing 8 concentration wells, 1 inlet for cell supply, 1 outlet for waste collection, and 2 traps which contain combs that can patch 20 cells per experiment (for a total of 40 cells per pattern). The inlet wells contain intracellular solution (in mM: CsCl 140, $CaCl_2$ 1, $MgCl_2$ 1, EGTA 11, and HEPES 10, pH 7.2 with CsOH). The cells were suspended in extracellular solution. The 8 concentration wells contained MIDD0301 diluted in DMSO, then diluted in ECS with a final DMSO concentration of 0.1%. Cells are captured in the traps through a pulse of suction, then whole cell recording access is obtained through a second strong pulse of suction which breaks the membrane. Compound application is achieved by applying pressure onto the appropriate well. Cells are voltage clamped at a holding potential of -80 mV.

Quantification of airway inflammatory cells. 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 of the following antibodies: anti-mouse CD45 APC (1:1000, 30- F11, Affymetrix eBiosciences, San Diego, CA), FITC rat anti-mouse CD4 (1:500, RM4−5, BD Pharmingen, San Jose, CA), FITC anti-mouse F4/80 (1:200, M1/70 Affymetrix eBiosciences, San Diego, CA) and PE rat anti-mouse Siglec-F (1:500, E50-2440, BD Pharmingen, San Jose, CA). Flow cytometric studies were done using the BD FACS Calibur (BD Pharmingen, San Jose, CA). Dead cells were excluded using the live/dead propidium iodide viability stain (BD Pharmingen, San Jose, CA). Data was analyzed subsequently using Cell Quest pro software (BD Pharmingen, San Jose, CA). Gating strategies for the different markers and treatment groups are shown in Supporting Information. The 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 $CD4^+$, $F4/80^+$ and Siglec-F^+ cell populations in their respective gates were multiplied by the total inflammatory cell count (cells/mL) to obtain the differential cell population.

EdU staining. In separate treatment groups (100 mg/kg MIDD0301 and vehicle treated ova s/c mice), mice received a single i.p. injection of EdU (Invitrogen, Carlsbad, CA) at a dose of 100 mg/kg before ketamine/xylazine overdose. Mice were euthanized 4 hours after injection and lungs were harvested, formalin fixed, and paraffin embedded. 6 μm lung sections were mounted onto Fisher Superfrost Plus Slides. EdU staining was conducted using Click-iT™ EdU imaging kit (Invitrogen, Carlsbad, CA) per manufacturer's instructions. Briefly lungs were deparaffinized in Histoclear and rehydrated in graded ethanol. Tissue sections were washed twice with 3% bovine

serum albumin (BSA) in PBS and permeablized with 0.5% Triton X-100 in PBS for 20 min. The sections were again washed twice with 3% BSA in PBS and then incubated with a Click-iT™ reaction cocktail containing Click-iT™ reaction buffer, CuSO4, Alexa Fluor® 488 Azide, and reaction buffer additive for 30 minutes in the dark. The sections were washed once more with 3% BSA in PBS. For DNA staining, sections were washed once with PBS and then incubated with 5 µg/mL Hoechst 33342 for 30 min. The slides were then washed twice with PBS and cover slipped with Permount mounting media. All steps were carried out at room temperature.

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 were well fixed. The lungs were then isolated from the thoracic cavity and kept in 10% neutral buffered cold formalin for 48 hours at 4 ^{0}C. The left lobe was then sectioned transversely into two. Lung samples were dehydrated, paraffin embedded, and sectioned. 6 µm sections were placed onto positively charged slides, dewaxed with Histoclear (National Diagnostics), 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 20 minutes at room temperature. The slides were washed with distilled water, rinsed in acidic alcohol, and cover slipped with Canada balsam and methyl salicylate mounting media to obtain counterstained slides.[29, 30] The PAFS-stained slides were examined under an EVOS fluorescence microscope and images from random fields of the lung tissue 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.[29, 30] Scale bars obtained during image acquisition were used to scale the images.

Cytokine expression. Lungs were isolated, snap frozen in liquid nitrogen, and stored at -80 ^{0}C until cytokine analysis. Whole lung was homogenized in 200 μL of T-PER® tissue protein extraction reagent (Thermo Fisher Scientific Inc., Rockford, IL) containing 1x protease inhibitor cocktail using a hand-held tissue homogenizer. Homogenized lung samples were centrifuged at 10,000 RPM for 5 minutes to pellet cell/tissue debris. Tissue supernatant was collected for cytokine analysis using BD cytometric bead array mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, San Jose, CA) following manufacturer's instructions. Samples were analyzed using FACSCalibur™ (BD Bioscience, San Jose, CA) flow cytometry and CELLQuest™ Software and FCAP Array™ Software (BD Bioscience, San Jose, CA). Individual cytokine concentrations were indicated by their fluorescence intensities.

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 or Tukey 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

MIDD0301 effectively relaxes airway smooth muscle. The $GABA_AR$ α_4 and α_5 subunits were identified previously in human and guinea pig airway smooth muscle.[18] Furthermore, these $GABA_AR$ can be targeted with subtype selective ligands to mediate smooth muscle relaxation.[13, 19] We assessed the smooth muscle relaxation potency of MIDD0301 in guinea pig tracheal rings *ex vivo* contracted with substance P. Direct action of substance P on the neurokinin 1 receptor (NK_1R) can lead to a series of GPCR signaling events including Gq coupling, phospholipase C

activation, formation of IP_3, and DAG, with subsequent Ca^{2+} mobilization.[31] A mechanism of smooth muscle contraction involving a decrease in membrane K^+ permeability with subsequent membrane depolarization has also been previously proposed.[32] The activity of MIDD0301 is depicted in Figure 2.

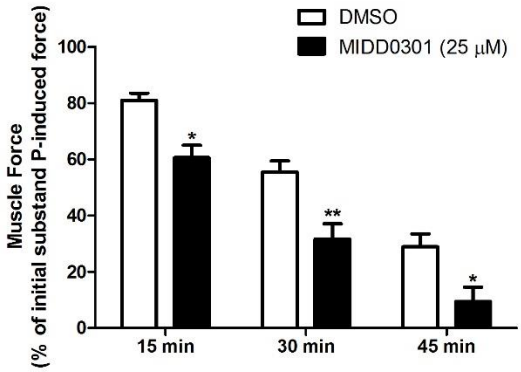


Figure 2. Muscle force in guinea pig airway smooth muscle contracted with 1 μM substance P. MIDD0301 (25 μM) induced a significant relaxation of substance P-contracted guinea pig tracheal rings compared to control (0.1% DMSO vehicle). Muscle force is expressed as a percent of the initial muscle remaining at various time points. A two-way ANOVA repeated measures analysis was used to determine significance with * and ** equals $p < 0.05$ and 0.01, respectively, compared to vehicle control ($n = 8$).

This study revealed a 25% relaxation of guinea pig smooth muscle contraction at 15 minutes following MIDD0301 application. The relaxation effect increased in magnitude over the 45 minute assay period. During the course of the assay, the contractile force induced by substance P diminished. MIDD0301 at 25 μM achieved smooth muscle relaxation similar to compound 2 at 50 μM using this assay.[13]

Oral administration of MIDD0301 reduced airway hyperresponsiveness. A cardinal measure of asthma severity is airway hyperresponsiveness (AHR) to broncho-constricting agents. A non-invasive airway mechanics instrument was used to quantify AHR in conscious, spontaneously breathing animals treated with increasing doses of methacholine. Consistent with previously published results,[12-14] ova s/c mice exhibited higher specific airway resistance (sRaw) values in comparison to control animals. The significance between sRaw values of control and ova s/c mice varied at different methacholine concentrations (Figure 3).

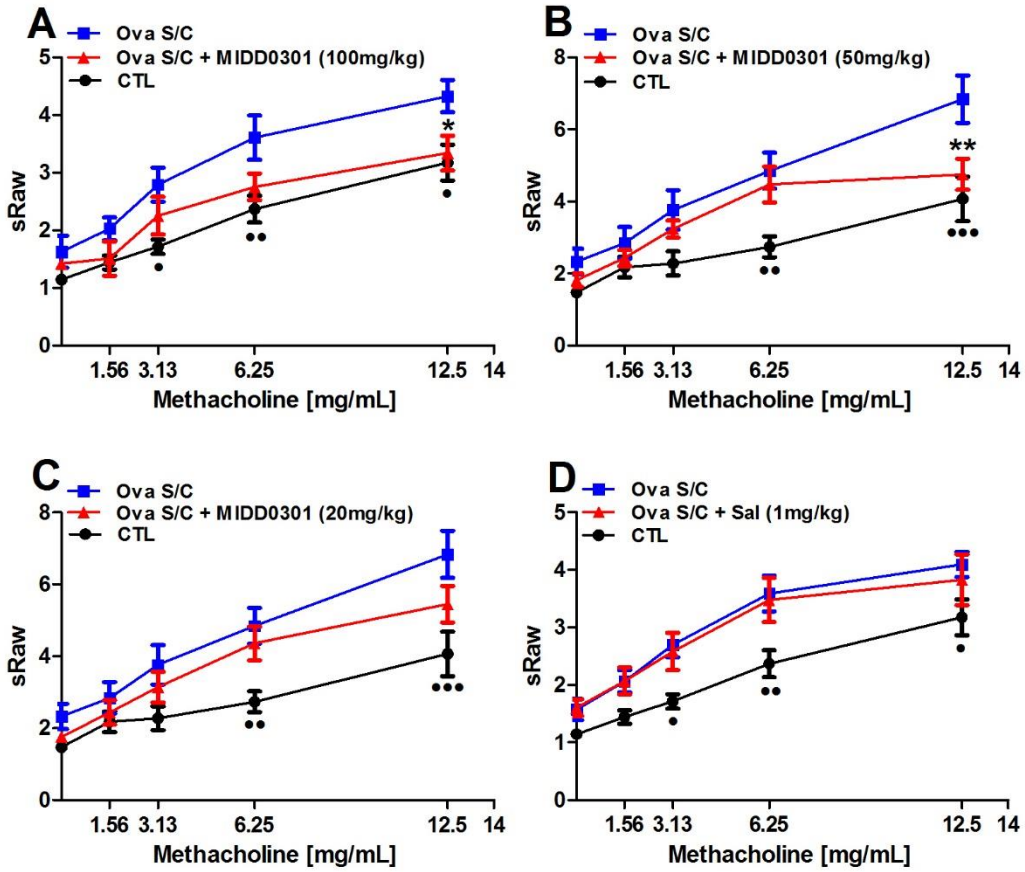


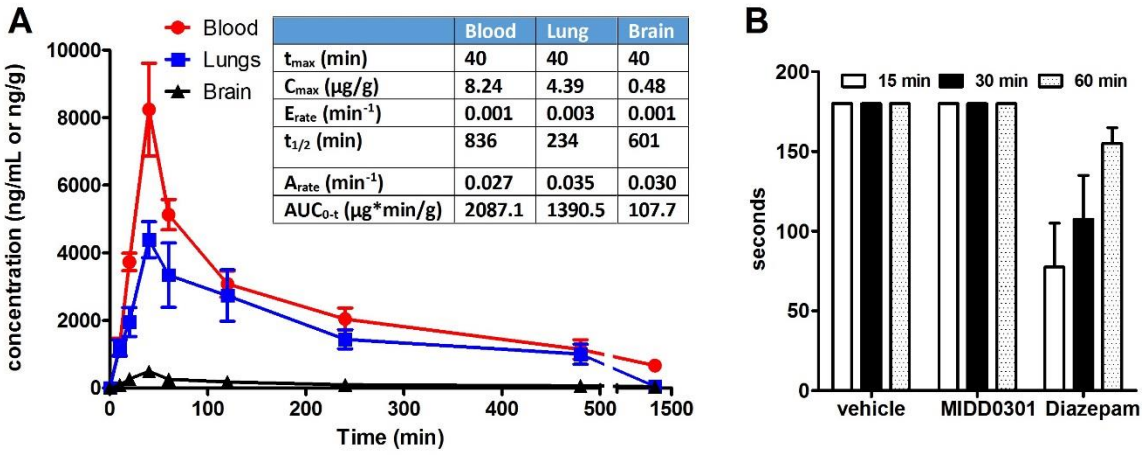
Figure 3. Effect of orally administrated MIDD0301 and salmeterol on airway hyperresponsiveness**.** Specific airway resistance (sRaw) was measured at increasing nebulized

dosages of methacholine by DSI's Buxco FinePointe noninvasive airway mechanics instrument (NAM). Ova s/c mice were administered vehicle or (A) 100 mg/kg, (B) 50 mg/kg, (C) 20 mg/kg of MIDD0301 via oral gavage b.i.d. for 5 days or (D) salmeterol also via oral gavage at 1 mg/kg b.i.d. for 5 days. Means ± SEM are presented for groups of 10 BALB/c mice. * and ** indicate $p < 0.05$ and $p < 0.01$ significance for the MIDD0301 group and •, ••, and ••• indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ significance between control mice compared to ova s/c mice.

Treatment of ova s/c mice orally with 100 mg/kg MIDD0301 b.i.d. significantly reduced AHR at 12.5 mg/ml nebulized methacholine (Figure 3, A). At this concentration, the significance between non-asthmatic mice (CTL) and treated asthmatic mice (ova s/c MIDD0301 (100 mg/kg) compared to the non-treated asthmatic mice (ova s/c) was the same ($p < 0.5$ for • and *). A similar effect was observed with the oral dosage of 50 mg/kg MIDD0301 b.i.d. (Figure 3, B), but with increased significance for control and treatment groups. Reducing the oral dose to 20 mg/kg reflected a downward trend for the sRaw value at 12.5 mg/ml methacholine, however, the effect did not reach statistical significance (p value of 0.507; Figure 3, C). Furthermore, we compared MIDD0301 with LABA salmeterol using the same route and frequency of administration at 1 mg/kg (Figure 3D). Interestingly, no AHR effect was observed for this approved inhaled asthma medication. In addition, a single high oral dose of salmeterol at 10 mg/kg was given acutely before the measurement without any significant effect on AHR (data not shown).

MIDD0301 is well distributed but with limited CNS exposure. The pharmacokinetic profile of MIDD0301 was investigated in mice over a period of 24 hours. The concentrations of MIDD0301

were quantified by LCMS/MS in blood, brain, and lung following a single oral administration of 25 mg/kg (Figure 4).



	Blood	Lung	Brain
t_{max} (min)	40	40	40
C_{max} (µg/g)	8.24	4.39	0.48
E_{rate} (min^{-1})	0.001	0.003	0.001
$t_{1/2}$ (min)	836	234	601
A_{rate} (min^{-1})	0.027	0.035	0.030
AUC_{0-t} (µg*min/g)	2087.1	1390.5	107.7

Figure 4. Pharmacokinetic profile of MIDD0301 in mouse blood, lungs, and brain. A) Time-dependent systemic distribution of MIDD0301 (25 mg/kg via oral gavage) and summary of calculated pharmacokinetic parameters; B) Sensorimotor coordination study using a rotarod apparatus with mice treated orally with 100 mg/kg MIDD0301 (n = 9). 5 mg/kg diazepam was administered i.p. for control purpose. The time that each treated mouse remained balanced on the rotating rod (15 rpm for up to 3 minutes) was recorded.

Within 40 minutes (T_{max}), MIDD0301 reached maximum absorption in blood and lung. The C_{max} for blood was 8.24 µg/g and 4.39 µg/g for lungs. The compound was well distributed in blood and lung with an AUC of 2087.1 and 1390.5 µg*min/g, respectively. The rate of elimination for MIDD0301 in the blood was slow at 0.001 min^{-1} resulting in a long half-life of 836 minutes. The elimination rate of MIDD0301 was somewhat faster in lung tissue. Further *in vitro* microsomal stability studies revealed that MIDD0301 is significantly more stable in human ($t_{1/2}$ = 1546

minutes) than in mouse ($t_{1/2}$ = 549 minutes) (Supporting Information). The mouse plasma protein binding of MIDD0301 is 88% (Supporting Information). Poor blood brain barrier penetration of MIDD0301 resulted in an extremely low AUC of 107.7 µg*min/g in the brain. Although this study showed negligible brain exposure to the compound, the absence of any possible adverse CNS effects such as sedation or ataxia caused by MIDD0301 was confirmed by a rotarod study (Figure 4, B). Here, groups of mice were treated orally with vehicle or 100 mg/kg of MIDD0301 and evaluated on a rotating rod for periods of three minutes at three different time points. All treated mice were able to successfully stay on the rotating rod during these time periods confirming the absence of sensorimotor inhibition by MIDD0301 in contrast to brain permeable positive control diazepam.

MIDD0301 is acting through the $GABA_AR$. Immune cells express multiple $GABA_AR$ subunits and react electrophysically when exposed to GABA and $GABA_AR$ ligands.[22-24] Recently we showed that $\alpha_4\beta_3\gamma_2$, and to a lesser extent $\alpha_5\beta_3\gamma_2$, subtype selective $GABA_AR$ ligands increase the current response of T lymphocytes in the presence of GABA.[12, 13] Accordingly, we investigated the dose dependent electrophysiological effect of MIDD0301 on $CD4^+$ T lymphocytes isolated from ova s/c mouse spleen using automated patch clamp (Figure 5).

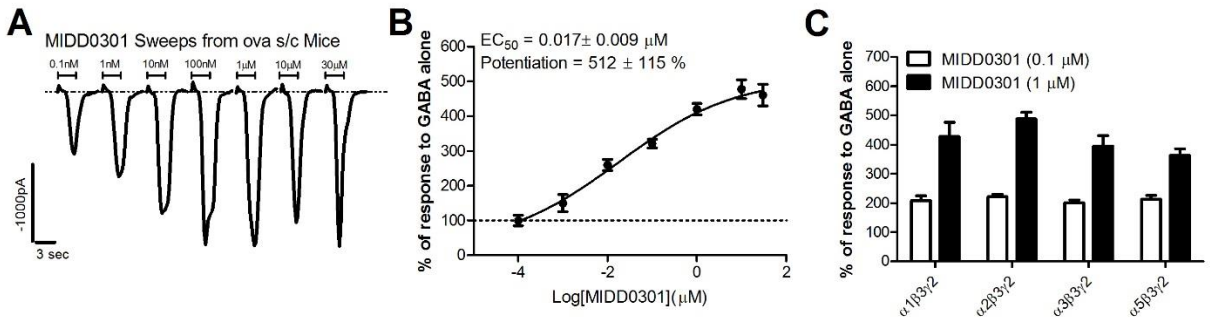


Figure 5. Induced electrophysiological responses by MIDD0301. A) Current responses of $CD4^+$ T lymphocytes isolated from ova s/c mice (n = 12) in the presence of 600 nM GABA and increasing concentration of MIDD0301 applied for 3 seconds, as determined by automated patch clamp. B) Normalized current responses of isolated $CD4^+$ T lymphocytes (ova s/c mice) in the presence of 600 nM GABA (100 %) and increasing concentrations of MIDD03101 for eight independent measurements with an n = 12. Data was fitted to a Y=Bottom + (Top-Bottom)/(1+10^((LogIC_{50}-X)*HillSlope)) to determine EC_{50} and top of the curve (potentiation); C) Average enhancement of current evoked to GABA by 0.1 μM or 1 μM of MIDD0301 using transiently transfected cells with α $GABA_AR$ subunits, as indicated, along with β_3 and γ_2L subunits measured by patch clamp. Data represent mean ± SEM with an n = 5.

MIDD0301 potentiated a current response in $CD4^+$ T cells at very low concentrations in the presence of 600 nM GABA, exhibiting a fast on-rate and rapid current decrease during the washout phase (Figure 5, A). The current change for MIDD0301 saturated at a concentration of 100 nM. The data showed an EC_{50} of 17 nM for MIDD0301 and maximal potentiation of 512%. Patch clamp measurements with cells transfected with various $GABA_AR$ subunits indicated that MIDD0301 is activating $GABA_ARs$ strongly among the alpha subtypes tested.

MIDD0301 has anti-inflammatory properties in the lung. Chronic airway inflammation is a hallmark feature of asthma and can be measured by quantification of immune cell subtypes in the bronchoalveolar lavage fluid (BALF). We analyzed BALF from vehicle and MIDD0301 treated mice by flow cytometry using differential counts for eosinophils, macrophages, and $CD4^+$ T cells, using Siglec F,[33] F4/80, and CD4 antibodies, respectively. Total inflammatory cells in BALF were

quantified with anti-CD45. The CD45 marker is also referred to as the leukocyte common factor, which is a 180-240 kD glycoprotein expressed on all hematopoietic cells except mature erythrocytes and platelets.[34] The results are depicted in Figure 6.

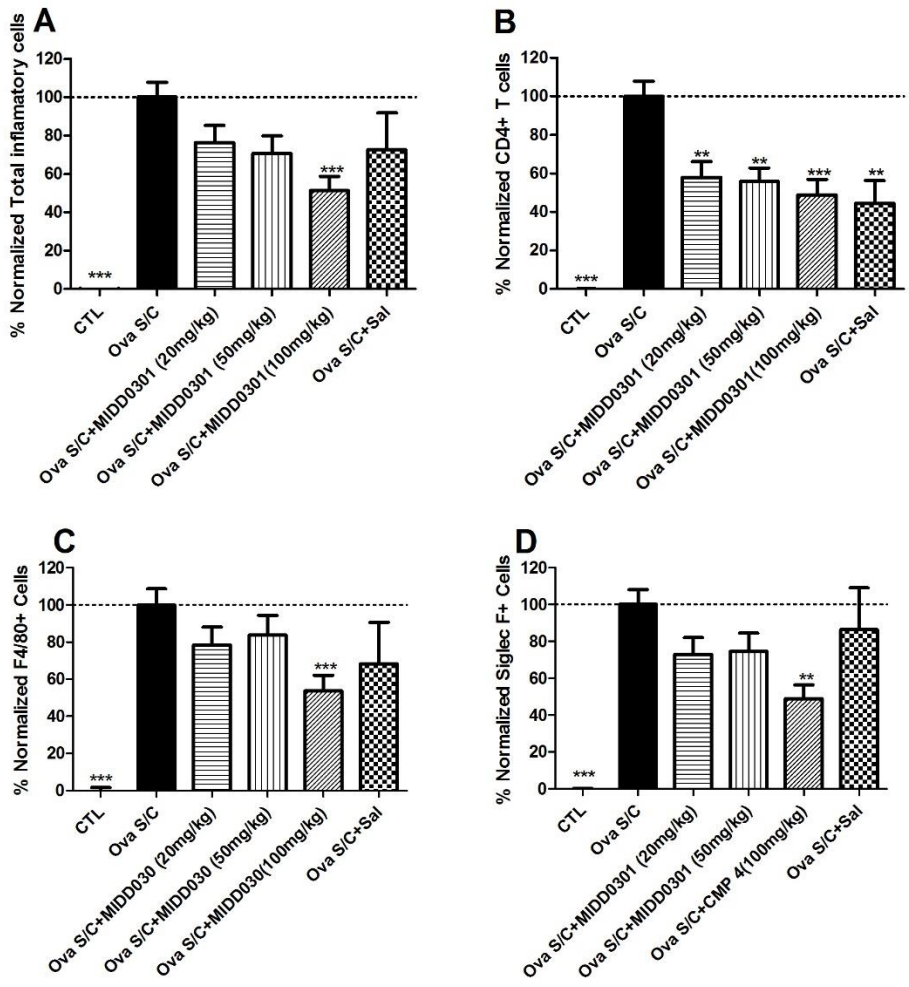


Figure 6. Effect of MIDD0301 and salmeterol on airway inflammatory cells. Groups of ova s/c mice were administered vehicle, MIDD0301 (20, 50 or 100 mg/kg), or salmeterol (1 mg/kg) via oral gavage b.i.d. for 5 days. BALF was harvested from each animal and used for (A) quantification of total inflammatory cells using anti-CD45 APC antibody and flow cytometry. (B) $CD4^+$ T cell, (C) $F4/80^+$ cell, and (D) Siglec F^+ cell populations were quantified by flow cytometry. Data represent mean ± SEM from 10 mice in each group. One way ANOVA was used to calculate

significance indicated as *, **, and *** for $p < 0.05$, $p < 0.01$, and $p < 0.001$ compared to vehicle treated ova s/c mice. The gated positive events are depicted in the Supporting Information.

We observed significant suppression of total inflammatory cells in BALF following 5 day oral administration with MIDD0301 at 100 mg/kg (Figure 6, A). Salmeterol at 1 mg/kg p.o. did not significantly change the leukocyte numbers. Efficacy of oral MIDD0301 treatment was also observed for the BALF Siglec F^+ cell population that include eosinophils/alveolar macrophages[35] and $F4/80^+$ cells that represent the murine macrophage population, though, only the 100 mg/kg dosage reduced their numbers significantly (Figure 6, C and D). In line with the sensitivity of $CD4^+$ T cells toward MIDD0301 determined by patch clamp, 20, 50 and 100 mg/kg oral MIDD0301 treatments significantly reduced the number of BALF $CD4^+$ T cells (Figure 6, B). Although salmeterol reduced BALF $CD4^+$ T cells (Figure 4 B), there was no significant effect observed for macrophage and eosinophil BALF populations (Figures 4, C and D).

Effects of MIDD0301 on the mouse lung. Lung inflammation is characterized by infiltration of leukocytes and proliferation of airway smooth muscle cells.[36] To visualize this effect in the mouse lung, a thymidine analogue EdU was i.p. injected into vehicle and MIDD0301 (100 mg/kg) treated ova s/c mice and control mice, allowing its incorporation into DNA during S phase of DNA replication. Mice were euthanized after four hours and lungs slices prepared following a standard histology protocol. The EdU labelled DNA was made visible via its conjugation with an Alexa Fluor® 488 azide using a copper catalyzed "Click" reaction.[37] The nuclei of all cells were counterstained with Hoechst 33342, and superimposed and individual images presented in Figure 7.

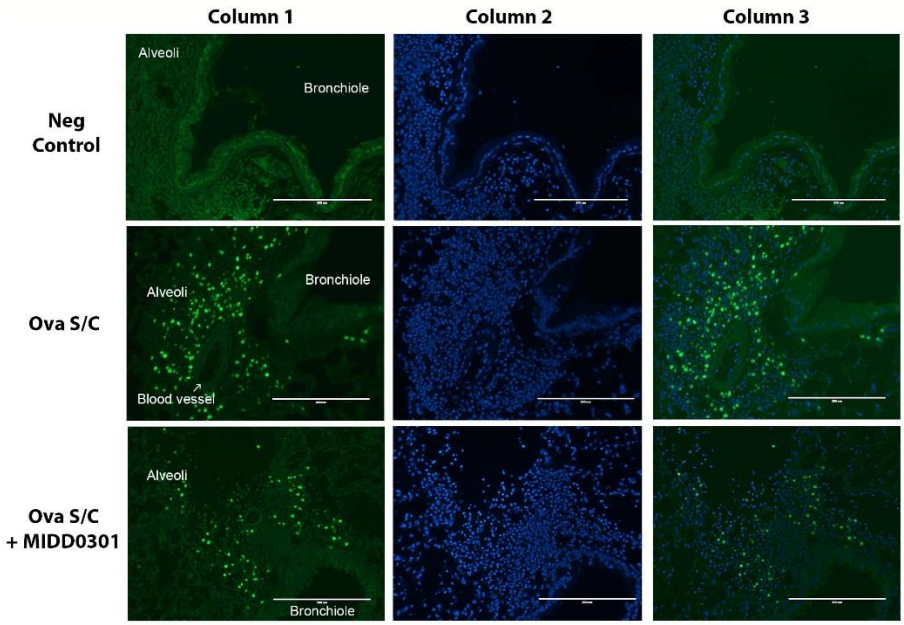


Figure 7. Cellular changes of the asthmatic mouse lung due to MIDD0301 treatment. Representative images of lungs from mice that were injected i.p. with EdU, a thymidine analog, and harvested four hours later. After standard histology processing, sections were treated with a fluorescent azide under "Click" chemistry conditions enabling conjugation of incorporated EdU to visualize cells that underwent the S phase during a four hour period (column 1). Slides were counterstained with Hoechst 33342 (column 2) and superimposed images are presented in column 3. Row 1 presents lung images of control mice. Row 2 depicts lung images of vehicle-treated ova s/c mice and row 3 images of MIDD (100 mg/kg) treated ova s/c mice.

EdU visualization of lung sections from control mice (non-asthmatic) revealed no cell staining except for faint non-specific background (Figure 7, column 1 and row 1). In contrast, lung sections from vehicle-treated ova s/c mice showed intensive staining in the alveolar region (Figure 7, column 1 and row 2). Cell layers of blood vessels (lamina and smooth muscle cells) and bronchiole (mucosa and smooth muscle cells) were not stained. Alveolar cells (pneumocytes) have not been

shown to proliferate quickly in the asthmatic lung, thus infiltrating alveolar leukocytes such as eosinophils, alveolar macrophages or monocytes were probably visualized.[38] Importantly, lungs sections from MIDD0301 treated ova s/c mice (Figure 7, column 1 and row 3) showed reduced cell staining in comparison to vehicle-treated ova s/c mice. Thus, the reduction of inflammatory cells observed in the BALF of MIDD0301 treated animals (Figure 6, A) is consistent with the reduction of alveolar inflammatory cells visualized by EdU.

Mucous metaplasia is unchanged for MIDD0301 treated asthmatic mice. Marked mucus accumulation is a key pathological feature of asthma resulting from mucus cell metaplasia (change in epithelial cell phenotype) and goblet cell hyperplasia (increase in goblet cell number). These histologic changes in the asthmatic lung can be visualized by staining sections with periodic acid fluorescent Schiff's stain (Figure 8).

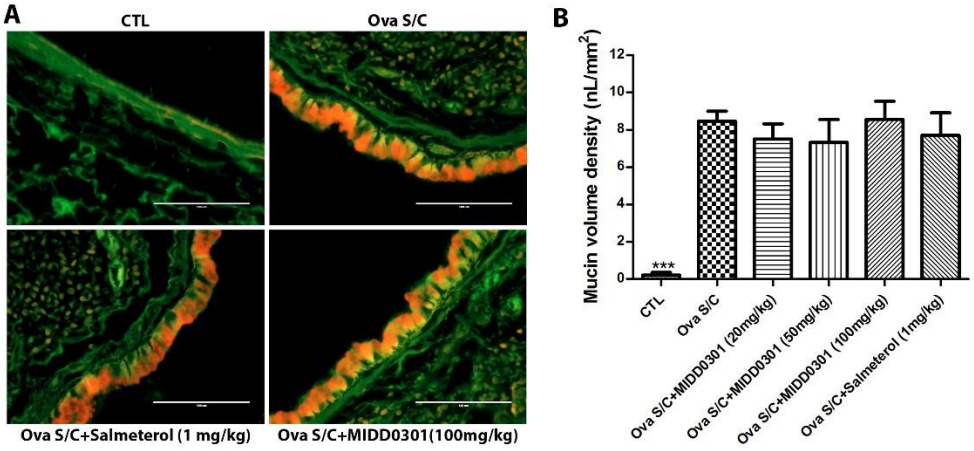


Figure 8. Effect of MIDD0301 and salmeterol on mucin production. A) Representative images of lung section of control mice (non-asthmatic) and ova s/c mice treated orally with vehicle, MIDD0301 (100 mg/kg, b.i.d. 5 days) or salmeterol (1 mg/kg, b.i.d. 5 days). Scale bar represents 100 μm. Slices were stained with periodic acid fluorescent Schiff's stain coloring airway

epithelium green and mucin red. B) Morphometric quantification of mucin volume density. Data represent mean ± SEM mucin volume density from six mice in each group.

The stained lung sections revealed significant increases in mucous metaplasia in ova s/c mice compared to control mice (Figure 8, A and B). MIDD0301 at 20, 50 and 100 mg/kg b.i.d. for 5 days did not produce a significant change in mucous production in the airways compared to ova s/c mice. Similarly, a 5 day b.i.d treatment with salmeterol at 1 mg/kg showed no effect on mucous levels in the airways.

Cytokine expression in mouse lung homogenate is significantly reduced by MIDD0301. Cytokines play critical functions and serve pleiotropic roles in asthma; hence concentrations of mouse TH_1 (IL-2, IFN-γ and TNFα), TH_2 (IL-4, IL-6 and IL-10), and TH_{17} (IL-17A) cytokines were quantified in lung homogenates using flow cytometry (Figure 9).[39]

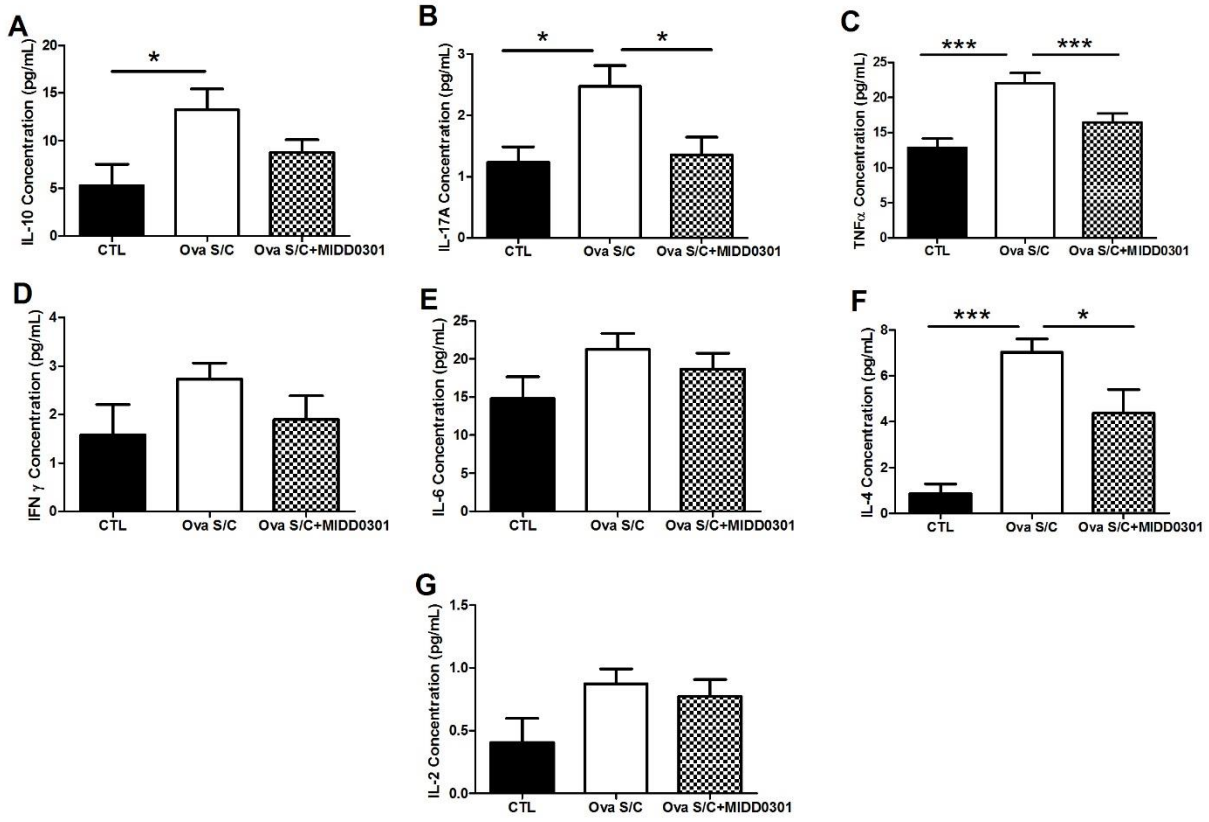


Figure 9. Effects of MIDD0301 on cytokine expression in the lung. Mouse Th1/Th2/Th17 cytokines were quantified in mouse tissue homogenates using the BD mouse Th1/Th2/Th17 cytometric bead array kit. Ova s/c mice were administered vehicle or 100 mg/kg MIDD0301 via oral gavage twice daily for 5 days. Data represent mean ± SEM from 10 mice in each group. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ significance determined by one-way ANOVA, compared to vehicle treated ova s/c mice.

Ova s/c mice had significantly higher concentrations of IL-4, IL-10, IL-17A, and TNFα compared to control mice (Figures 6A, 6B, 6C and 6F, $p<0.05$). The concentrations of IL-2, IL-6, and IFN-γ in lung homogenates of ova s/c mice were not significantly different from control mice (Figures 6C, 6D, 6E and 6C). Cytokine levels that were significantly increased in the lung of ova s/c mice

in comparison to the control mice, were in turn significantly reduced with the treatment of MIDD0301, except for IL-10. The cytokine concentrations that did not change between the control and ova s/c mice were not altered significantly by MIDD0301 treatments.

DISCUSSION

Among the various alpha $GABA_AR$ subunits, only α_4 and α_5 subunit containing $GABA_AR$ are expressed in airway smooth muscle.[18] Immune cells also express receptors containing these two alpha $GABA_AR$ subunits in addition to α_2 and α_3.[13] Thus, the presence of an overlapping subset of discrete $GABA_ARs$ comprising these alpha subunits enables a novel drug design strategy to target two hallmarks of asthma: airway smooth muscle constriction and lung inflammation. To prove this rationale, prototype $GABA_AR$ ligands possessing $\alpha_4\beta_3\gamma_2$ and the $\alpha_5\beta_3\gamma_2$ efficacy were shown to alleviate both of these asthma symptoms *in vitro* and *in vivo*.[13, 14] Historically, $GABA_AR$ ligands based on the benzodiazepine scaffold have been developed to treat various CNS disorders such as anxiety and seizures. A critical innovation of MIDD0301, and earlier $GABA_AR$ ligands developed for asthma, is their altered chemical structure that restricts brain exposure but facilitates pharmacological activity to readily permeable lung tissues. Pharmacokinetic studies have confirmed negligible concentrations of MIDD0301 in the brain, which is protected by tight junctions between endothelial cells creating the blood brain barrier. The predicted logBB of -0.14 for MIDD0301, based on its total polar surface area (70.3 A^2) and logP (4.2), is consistent with this *in vivo* observation.[40] In addition, MIDD0301 caused no sensorimotor impairment in rotarod studies, as would be observed if CNS adverse effects were present. Furthermore, this study also showed that MIDD0301 did not diminish skeletal muscle coordination necessary for the animal to

stay balanced on the rotating rod. This finding was observed for midazolam, which has been shown to block inactivated Na channels in skeletal muscle fibers.[41]

Importantly, MIDD0301 has been shown to relax airway smooth muscle using *ex-vivo* substance P mediated pre-contracted guinea pig tracheal rings. Treatment with 25 μM MIDD0301 was sufficient to partially relax muscle constriction within 15 minutes. Earlier experiments with an analog of MIDD0301 have shown that the actual concentration of compound in the trachea is only 10% of the organ bath concentration due to limited passive diffusion;[13] thus MIDD0301 is pharmaceutically active at single digit micromolar concentration in this assay. The increased potency of MIDD0301 in comparison to previous $GABA_AR$ modulators developed for asthma was also observed by the alleviation of the ova s/c induced asthma phenotype in BALB/c mice. Oral treatment of MIDD0301 at 50 mg/kg b.i.d. for 5 days was sufficient to overcome induced AHR at a 12.5 mg/kg dose of nebulized methacholine. The therapeutic effective dose range of MIDD0301 is between 20 and 50 mg/kg because 20 mg/kg p.o. did not reduce AHR significantly after the 5 day b.i.d. treatment.

Another critical feature of asthma is persistent goblet cell hyperplasia, mucus cell metaplasia, and mucus hypersecretion.[42] Mucus hypersecretion from hyperplastic goblet cells causes mucus plugging, particularly in the peripheral airways and is a major pathologic finding in asthma mediated deaths.[43, 44] Ovalbumin and nicotine exposure have shown to induce mucus secretion and increased expression of glutamic acid decarboxylase (GAD) and $GABA_AR$ subunits in lung epithelia cells.[21, 45-47] In these studies, bicuculline, a $GABA_AR$ antagonist that also blocks small-conductance calcium-activated potassium channels,[48] has been shown to reduce GABA-induced transmembrane current and mucin 5A expression in lung epithelia cells, as well as mucus secretion and AHR. The administration of imidazobenzodiazepine $GABA_AR$ modulators,

however, did not alter the mucus production in the lung of ova s/c mice at 100 mg/kg.[12, 13] Similar results were obtained with MIDD0301 when administrated at 20, 50 or 100 mg/kg b.i.d. for 5 days.

A major pathological feature of persistent asthma is chronic allergic inflammation leading to airway eosinophilia.[49] We demonstrated a significant reduction for BALF eosinophil numbers in ova s/c mice following MIDD0301 treatment with 100 mg/kg twice daily for 5 days. In asthma, airway macrophages are one of the major cell types involved in the chronic inflammatory process and can be divided into three classes: bronchial macrophages (BMs), alveolar macrophages (AMs), and interstitial macrophages (IMs).[50] MIDD0301 at 100 mg/kg b.i.d. for 5 days reduced the number of macrophages in the BALF of ova s/c mice. Cell staining with EdU confirmed the reduction of cells the alveoli region of the lung, which might be recruited eosinophils, alveolar macrophages or monocytes, because resident alveolar macrophage proliferate very slowly or not at all.[51] Further studies will be conducted to confirm this hypothesis.

In contrast to compound 2, inflammation is reduced by MIDD0301 by direct interaction with $CD4^+$ T cells as demonstrated by modulation of its transmembrane current in the presence of GABA. We hypothesize that this effect is mediated by the ability of MIDD0301 to activate the α_2 subunit containing $GABA_AR$, which has been identified on $CD4^+$ T cells.[52] *In vivo*, airway $CD4^+$ T cell numbers were significantly reduced in ova s/c mice following treatment with 20 mg/kg MIDD0301 b.i.d. for 5 days. In asthma, $CD4^+$T cells produce several TH_2 interleukins such as IL-4, IL-5, and IL-13.[53] As expected, IL-4 levels in lung homogenates were reduced with the treatment of MIDD0301 in comparison to vehicle-treated ova s/c mice. IL-4 is a key cytokine in the development of allergic inflammation and major mediator of isotype switching and secretion of IgE, which in turn promotes eosinophil transmigration across endothelium, mucus secretion, and differentiation of TH_2 lymphocytes leading to cytokine release.[54] Activated $CD4^+$ T cells also

produce IL-17, which mediates multiple aspects of asthma pathogenesis and has been found in extremely high levels in sputum and bronchial biopsies of patients with severe asthma.[55] Importantly, we demonstrated that MIDD0301 treatment significantly reduced IL-17A levels in lung homogenates of ova s/c mice. Low IL-17 levels might be one reason for decreased lung TNF-α levels observed for MIDD0301 treated ova s/c mice due to the fact that IL-17 has been shown to stimulate TNF-α expression by macrophages.[56] The lower number of macrophages found in BALF of MIDD0301 treated ova s/c mice might contribute to the reduction of TNF-α. Low TNF-α levels are important therapeutically, because emerging evidence suggests that this pro-inflammatory cytokine plays an important role in severe refractory disease and in many aspects of the airway pathology of asthma.[57] The modulation of other cytokines such as IFN-γ, IL-6, and IL-2 is unclear because the ova s/c phenotype did not demonstrate levels significantly different from control mice. Importantly, the anti-inflammatory cytokine IL-10 expression in the lung was not significantly altered by MIDD0301 treatment.

In conclusion, following earlier development of $\alpha_4\beta_3\gamma_2$ and the $\alpha_5\beta_3\gamma_2$ selective $GABA_AR$ ligands to treat asthma, we now report a more potent, orally available asthma drug candidate MIDD0301. This compound has significantly improved anti-inflammatory properties in the lung in addition to its ability to rapidly relax constricted airway smooth muscle at low concentrations. Current studies are focused on better understanding the respiratory immune modulating effects of this novel class of compounds in addition to future IND enabling studies with MIDD0301.

Supporting Information. The Supporting Information includes Supplemental Experimental Procedures and six figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding Sources. 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.), the Foundation for Anesthesia Education and Research (G.T.Y.).

Abbreviation: $GABA_AR$, $GABA_A$ receptor; AHR, airway hyperresponsiveness; CNS, central nervous system; NMR, nuclear magnetic resonance; HRMS, high resolution mass spectrometry; ECS, external cell solution; ICS, internal cell solution; DMSO, dimethylsulfoxide; BALF, bronchoalveolar lavage fluid; GABA, gamma aminobutyric acid; sRaw, specific airway resistance; PK, pharmacokinetic studies; LABA, long acting β2 adrenergic receptor agonists; NK_1R, neurokinin 1 receptor; BM, bronchial macrophages; AM, alveolar macrophages; IM, interstitial macrophages; PAFS, Periodic Acid Fluorescent Schiff's Stain.

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

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