

Nebulized MIDD0301 Reduces Airway Hyperresponsiveness in Moderate and Severe Murine Asthma Models

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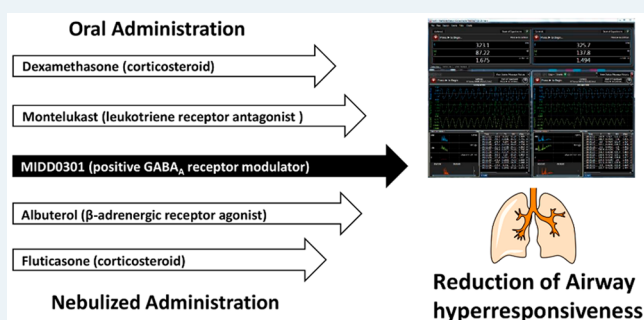
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ABSTRACT: We report the relaxation of methacholine-constricted airways with nebulized MIDD0301, a positive allosteric γ -aminobutyric acid type A receptor ($GABA_A$ R) modulator. The therapeutic efficacy of nebulized MIDD0301 in reducing airway resistance was investigated in spontaneous breathing mice using a whole-body plethysmograph and in unconscious mice using a forced oscillation technique. Prophylactic nebulized MIDD0301 reduced subsequent methacholine-induced bronchoconstriction in ovalbumin and house dust mite allergic asthma models and in normal mice. Nebulized MIDD0301 exhibited comparable or better therapeutic potency compared to nebulized albuterol and oral montelukast. Prophylactic nebulized MIDD0301 was also effective in reducing bronchoconstriction, comparable to nebulized albuterol or fluticasone, in a steroid resistant asthma mouse model induced by intratracheal installation of lipopolysaccharide and interferon- γ . Oral dexamethasone was ineffective in this model. Nebulized MIDD0301 was also effective in reversing bronchospasm when dosed after methacholine challenge comparable to albuterol. Pharmacokinetic studies showed that about 0.06% of nebulized MIDD0301 entered the mouse lung when using a whole body plethysmograph and therapeutic levels were sustained in the lung for at least 25 min. Consistent with previous reports on orally dosed MIDD0301, high doses of nebulized MIDD0301 resulted in minimal brain exposure and thus no observable adverse sensorimotor or respiratory depression effects occurred. In addition, no adverse cardiovascular effects were observed following 100 mg/kg i.p. dosing. These results further demonstrate that charged imidazodiazepine MIDD0301 can selectively target lung $GABA_A$ R without adverse motor, cardiovascular, or respiratory effects and inhaled dosing is effective in reducing bronchoconstriction in allergen and infectious lung inflammation.

KEYWORDS: asthma, MIDD0301, bronchodilation, airway hyperresponsiveness



INTRODUCTION

During acute asthma exacerbations, airway narrowing caused by airway smooth muscle (ASM) contraction (bronchospasm) occurs rapidly in response to allergens, irritants, or other stimuli such as exercise, cold air, and anxiety. Rapid onset ASM relaxing agents (bronchodilators) are used for immediate bronchospasm relief, and combinations of β -adrenergic receptor agonists (BAA), anticholinergic agents, leukotriene receptor antagonists, corticosteroids, and biologics are used to maintain airway function and control underlying asthmatic inflammation. Asthma is a very heterogeneous disease, reflected in a spectrum of phenotypes and endotypes, including paucigranulocytic, eosinophilic, neutrophilic, and mixed-granulocytic inflammation,¹ which have variable responses or resistance to existing pharmacotherapy. Targeting gamma-butyric acid type A receptors ($GABA_A$ Rs) in the lung represents an alternative asthma drug strategy in addition to other approaches.² $GABA_A$ Rs are well-characterized ligand

gated chloride ion channels known for their expression on neurons and response to inhibitory neurotransmitter GABA and other ligands.³ Recently, functional $GABA_A$ Rs have been identified on non-central nervous system (CNS) cell types, such as ASM⁴ and inflammatory cells.⁵ We have developed subtype selective $GABA_A$ R allosteric ligands that relax constricted rodent and human ASM *ex vivo*.^{6–12} *In vivo* studies with orally dosed asthmatic mice demonstrated reduced inflammation and relaxation of methacholine-induced bronchospasm.^{6–9,11}

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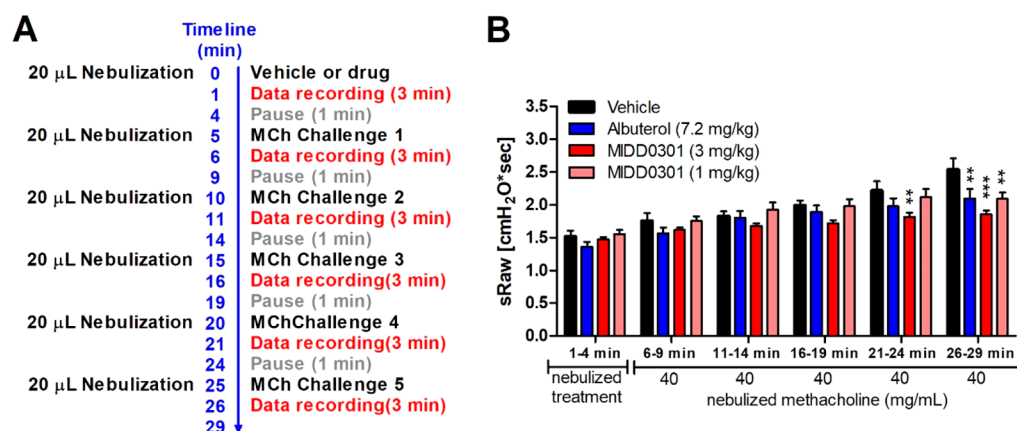


Figure 1. Pharmacological effect of MIDD0301 in reducing methacholine-induced airway constriction (prophylaxis). (A) Protocol to determine airway hyperresponsiveness using the Buxco FinePointe noninvasive airway mechanics instrument (NAM). (B) Specific airway resistance (sRaw) was measured using female C57BL/6J-129S6 hybrid mice after nebulization of vehicle (PBS) or drug diluted in vehicle followed by nebulized challenge doses of methacholine. Data are depicted as means $\pm$ SEM of animal groups having $n = 10$. ** and *** indicate $p < 0.01$ and $p < 0.001$ significance between vehicle- and drug-treated animals for each individual methacholine challenge determined by two-way ANOVA.

A growing body of research describes GABA as an important modulator in cells of the immune system,¹³ which is present in nanomolar concentrations in the blood.¹⁴ Our group has demonstrated that nanomolar concentrations of GABA_AR allosteric ligands and GABA are sufficient to change the transmembrane current of isolated mouse CD4⁺ T cells.⁷ Oral administration of allosteric GABA_AR ligands reduced the number of lung inflammatory cells in ovalbumin-sensitized and challenged asthmatic mice. Cytokines, such as IL-17, IL-4, and TNF α , were also downregulated in the lungs.⁶

Respiratory diseases such as asthma and COPD are often treated with inhaled BAA and steroids to achieve high concentrations locally in the lung and minimize systemic exposure. However, about 80–90% of an inhaled medication is likely to be swallowed.¹⁵ Adverse systemic effects of BAA include increased heart rate, palpitation, vasodilation, and reflex tachycardia.¹⁶ To prevent adverse systemic effects, “antedrugs” with limited systemic half-lives were developed.¹⁷ This strategy was also employed to reduce the adverse effects of inhaled corticosteroids,¹⁸ which include adrenal atrophy, osteoporosis, steroid-induced myopathy, osteonecrosis, fluid retention, edema, hypertension, arrhythmias, and dermatological, ophthalmologic, gastrointestinal and neuropsychiatric effects.¹⁹ In contrast, orally administered allosteric GABA_AR modulator MIDD0301 exhibited no observed systemic adverse effects following repeated dosing of 200 mg/kg for 28 days.²⁰ A 40-fold lower dose of prednisone caused weight loss and reduced the size of the spleen and thymus.

Here, we demonstrated that MIDD0301, in addition to its ability to reduce asthma symptoms when administered orally,⁶ rapidly reduced bronchoconstriction when delivered by inhalation. The onset of action and effective doses were comparable to BAA albuterol in allergen-induced murine asthma models. Repeated inhaled nebulized doses of MIDD0301 achieved bronchodilation at doses lower than single treatments. Cardiovascular, CNS, and respiratory toxicity studies confirmed the safety of inhaled MIDD0301 at exposure 50-fold greater than its therapeutic dose.

RESULTS AND DISCUSSION

The studies presented herein offer further characterization of drug candidate MIDD0301 as an inhaled medication for rapid

relief of bronchospasm. The described pharmacological effects of nebulized MIDD0301 in murine lung inflammation induced by diverse allergens and bacterial and viral mediators (and steroid resistant inflammation) are consistent with future clinical evaluation of MIDD0301 as a first-line drug for use across asthma inflammatory endotypes. Despite the availability of several drugs for asthma symptom control, the disease progresses in many patients, prompting increased dosing, the use of drug combinations, or the use of stronger medications with greater risks of adverse effects. By safely targeting GABA_AR in the lung, MIDD0301 offers a novel mechanism of action and provides an important therapeutic alternative to existing drugs that are limited by safety liabilities, restricted efficacy, and drug tolerance.

We quantified the efficacy of MIDD0301 by its ability to reduce methacholine-induced airway hyperresponsiveness in mice using a whole-body plethysmograph. Following the protocol depicted in Figure 1A, the vehicle or drug (MIDD0301 or albuterol) was nebulized and the specific airway resistance (sRaw) recorded for 3 min. After 1 min of acclimatization, methacholine (40 mg/mL, 20 μ L) was nebulized followed by 3 min of data acquisition and a 1 min acclimatization phase. This procedure was repeated five times, and average sRaw values for albuterol and MIDD0301 were recorded (Figure 1B).

sRaw increased with successive constant methacholine challenges in female C57BL/6J-129S6 (F1) hybrid mice from 1.5 to 2.5 cmH₂O*s. Experiments with female C57BL/6J mice resulted in constant sRaw values at methacholine concentrations up to 60 mg/mL (data not shown). Nebulized MIDD0301 (3 mg/kg) significantly reduced sRaw of C57BL/6J-129S6 mice during the fourth and fifth methacholine challenges and was more effective than 7.2 mg/kg albuterol at these challenges. Albuterol (7.2 mg/kg) and MIDD0301 (1 mg/kg) were similarly effective in reducing sRAW during the fifth methacholine challenge, reflecting greater potency of MIDD0301 in reducing methacholine-induced airway constriction compared to nebulized albuterol in normal, spontaneously breathing mice without lung inflammation.

Next, we investigated the pharmacological activity of MIDD0301 using mouse asthma models where lung inflammation was induced by either ovalbumin (OVA) or

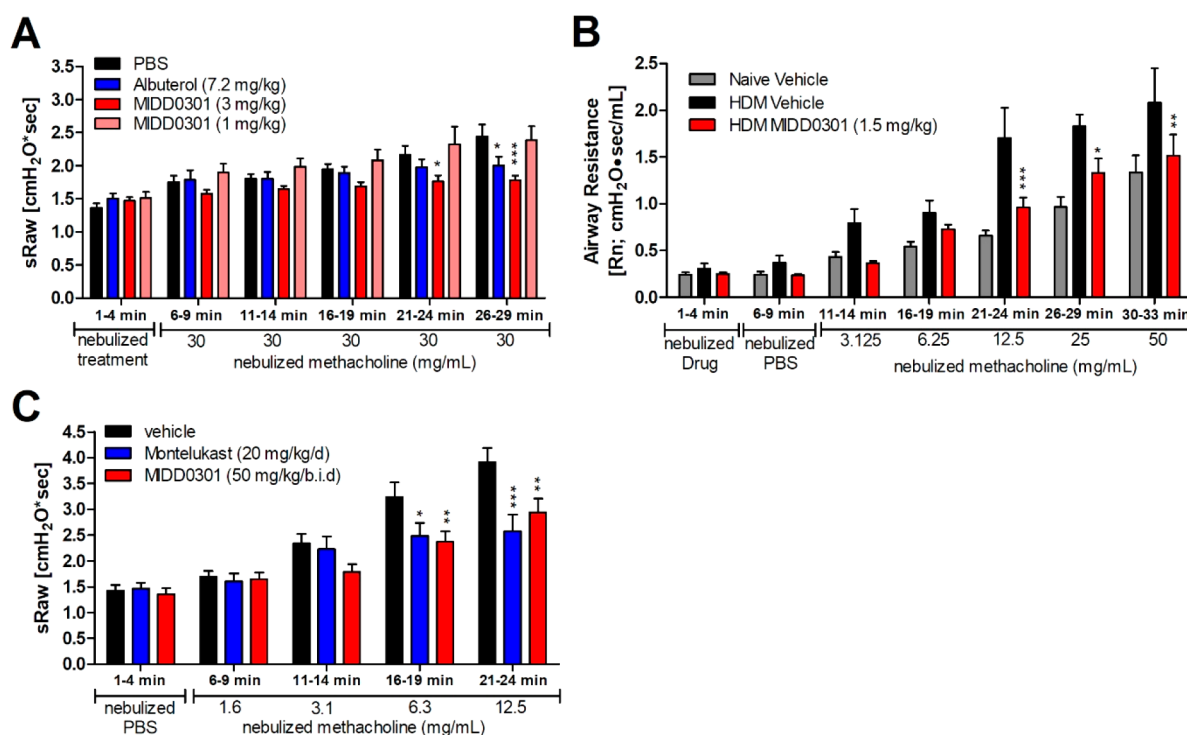


Figure 2. Pharmacological activity of MIDD0301 in allergen-induced asthmatic mice. (A) Female C57BL/6J-129S6 hybrid mice were challenged intranasally with 1 mg/kg ovalbumin for five consecutive days ($n = 11$). Specific airway resistance (sRaw) was measured using a NAM instrument after a single nebulized dose of vehicle or vehicle plus drug, followed by nebulized methacholine challenge. (B) Male C57BL/6J mice were challenged intranasally with HDM daily for 3 weeks ($n = 10$). Specific airway resistance (Rn) was measured using a forced oscillation technique (flexiVent) after a single nebulization of vehicle or vehicle plus drug, followed by nebulized methacholine challenge. (C) Male BALB/c mice were sensitized (days 0, 7, and 14) and challenged intranasally (days 22–27) with OVA ($n = 14$). Drugs were administered orally for 5 days during the intranasal OVA challenge. Specific airway resistance (sRaw) was measured using a NAM instrument after nebulized methacholine challenges. Data are shown as means $\pm$ SEM. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ significance between vehicle- and drug-treated animals for each individual methacholine challenge determined by two-way ANOVA.

house dust mite (HDM) allergen sensitization. sRaw values were determined after nebulized methacholine challenge by whole-body plethysmography (Buxco), and airway resistance (Rn) was determined by the forced oscillation technique (flexiVent). The procedure shown in Figure 1A was followed for nebulized albuterol and MIDD0301. Data collected using OVA-sensitized C57BL/6J-129S6 hybrid mice are presented as sRaw in Figure 2A. Figure 2B shows airway resistance values of naïve and HDM-sensitized C57BL/6J mice treated with MIDD0301 or vehicle. Evaluation of oral asthma drug montelukast and MIDD0301 using the OVA model in BALB/cJ mice is depicted in Figure 2C.

OVA-sensitized C57BL/6J-129S6 hybrid mice pretreated with nebulized vehicle exhibited sRaw values increasing from 1.4 to 2.4 cmH₂O*s during a course of five sequential challenges of 30 mg/mL methacholine (Figure 2A). Prophylactic dosing of 3 mg/kg MIDD0301 significantly reduced sRaw at both the fourth and fifth methacholine challenge points and gave responses greater than 7.2 mg/mL nebulized albuterol. The lower dose of 1.0 mg/kg MIDD0301 was not effective in reducing sRaw at any of the albuterol challenges.

In comparison to OVA sensitization, HDM allergen sensitization caused greater influx of eosinophils and neutrophils in the BALF, higher levels of IL-4 and IL-10, and increased subepithelial collagen in C57BL/6 mice.²¹ We challenged C57BL/6J mice daily with intranasally administered HDM for 3 weeks, which resulted in a significant increase of lung resistance measured by flexiVent at 12.5, 25, and 50 mg/

mL nebulized methacholine. The airway resistance increased from 0.3 to 2.1 cmH₂O*s/mL during the five graded methacholine applications. 1.5 mg/kg MIDD0301 nebulized before the methacholine challenge reduced airway resistance at the 12.5, 25, and 50 mg/mL methacholine doses (Figure 2B). The absolute airway resistance of HDM-challenged and MIDD0301-treated mice was comparable to the naïve C57BL/6J mice for the 12.5 and 50 mg/mL methacholine doses.

It has been reported that OVA sensitization results in more robust AHR in BALB/c compared to C57BL/6 mice; conversely, peribronchial eosinophilia was more pronounced in the C57BL/6 compared to BALB/c mice.²² We used the BALB/c model to compare AHR following oral treatment with either leukotriene receptor antagonist montelukast or MIDD0301. Here, we observed a change of sRaw from 1.5 to 3.8 cmH₂O*s during graded methacholine challenges in vehicle-treated mice. Treatment with montelukast (20 mg/kg/day) for five consecutive days significantly reduced AHR at the 6.25 and 12.5 mg/mL methacholine challenges (Figure 2C). MIDD0301 treatment (50 mg/kg/b.i.d.) over the same 5 day period caused a similar significant reduction of sRaw. We previously reported the characterization of alleviated lung inflammation (inflammatory cell numbers and cytokine levels) in this model after repeated oral dosing of MIDD0301.⁶

The relief of acute bronchospasm by inhaled drug treatment (in contrast to the previous prophylactic study design) was investigated with A/J mice, which display pronounced AHR to

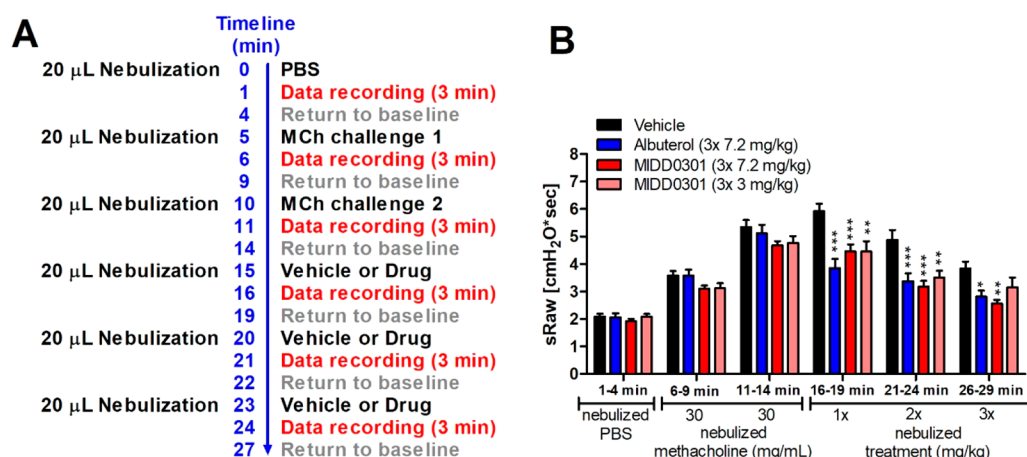


Figure 3. Acute relief of methacholine-induced bronchospasm with MIDD0301 or albuterol treatment. (A) Protocol to determine AHR using the NAM instrument. (B) sRaw was measured using female A/J mice first challenged with methacholine followed by treatment with nebulized vehicle or indicated drug. Data are depicted as means $\pm$ SEM of $n = 10$. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ significance between vehicle- and drug-treated animals using two-way ANOVA.

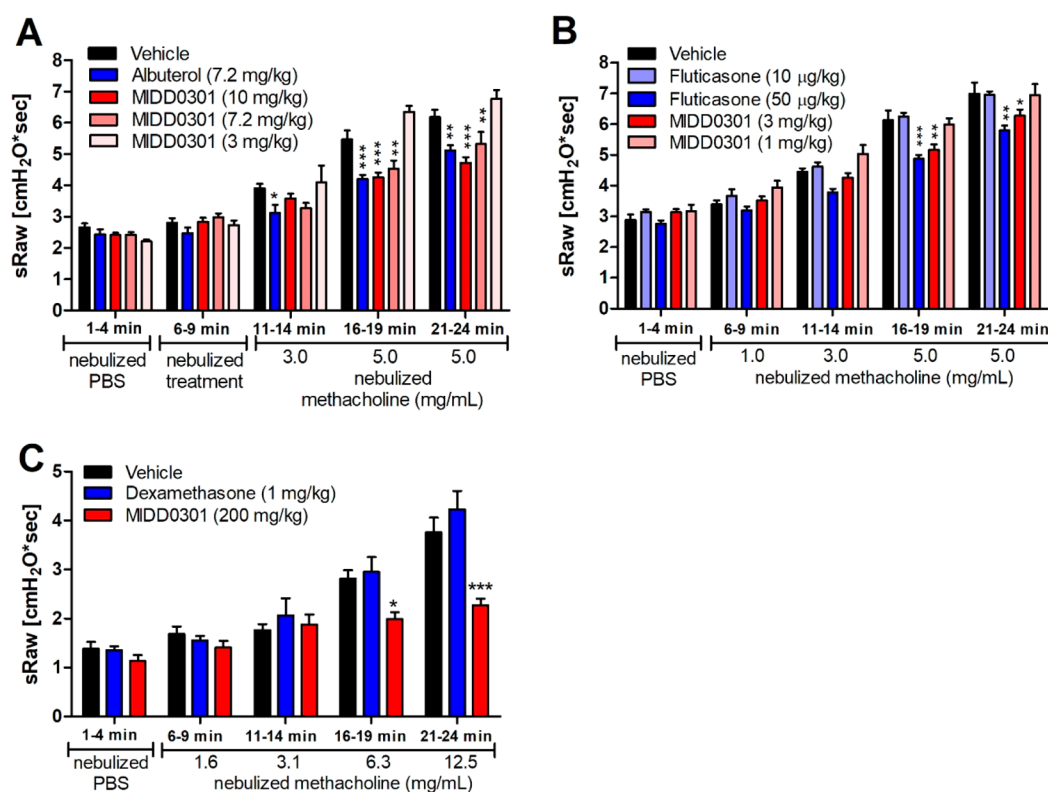


Figure 4. Pharmacological effect of MIDD0301 in a steroid resistant asthma model. (A) Female Swiss Webster mice were challenged intratracheally with LPS and $\text{INF}\gamma$ 1 day before the measurement. sRaw was measured repeatedly after nebulization of vehicle, followed by drug in vehicle and nebulized methacholine using the NAM instrument. (B) Nebulized fluticasone and MIDD0301 were administered to female Swiss Webster mice daily for 3 days followed by intratracheal instillation of LPS and $\text{INF}\gamma$ on the second to last day. One hour after the last administration of vehicle or drug, mice were exposed to nebulized PBS and graded doses of methacholine each followed by sRaw measurements. (C) Dexamethasone was administered orally for three consecutive days and MIDD0301 for five consecutive days to female BALB/c mice followed by intratracheal administration of LPS and $\text{INF}\gamma$ on the second to last day. One hour after the last administration of vehicle or drug in vehicle on the last day, mice were exposed to nebulized PBS followed by doses of methacholine. Data are depicted as means $\pm$ SEM of $n = 10$. *, **, and *** indicate $p < 0.05$, $p < 0.01$, and $p < 0.001$ significance between vehicle- and drug-treated groups determined by two-way ANOVA.

methacholine challenge in the absence of lung inflammation induced by allergen.²³ In the treatment study design outlined in Figure 3, animals were challenged first with nebulized methacholine followed by nebulized treatment with MIDD0301 or albuterol.

After two nebulized methacholine (30 mg/mL, 20 μ L) challenges, sRaw reached 5.4 $\text{cmH}_2\text{O} \cdot \text{s}$. Subsequent nebulized vehicle treatments resulted in sRaw values ranging from 5.8 to 3.9 $\text{cmH}_2\text{O} \cdot \text{s}$, which were all significantly elevated from the sRaw at the initial vehicle administration. Thus, elevated AHR

persisted for at least 15 min after the methacholine challenge. Mailhot-Larouche et al. reported bronchoconstriction for at least 10 min after methacholine challenge in BALB/c mice.²⁴ The first application of albuterol (7.2 mg/kg) reduced AHR, confirming its activity as a fast-acting bronchodilator. Further AHR reductions were observed during the subsequent second and third doses. MIDD0301 (7.2 mg/kg) also reduced AHR during all three doses similar to albuterol. Although these compounds mediate their pharmacological effect through different receptors, G-protein-coupled receptor BAA versus ion-channel GABA_AR, their onset of action is similar. Relaxation of ASM *ex vivo* by MIDD0301 was observed at least 15 min following administration.^{6,10} In a similar assay, the reported onset of action of long-acting BAA salmeterol was 19.5 min, whereas albuterol reduced bronchoconstriction as quickly as 1.8 min.²⁵ Herein, we demonstrated *in vivo* that MIDD0301 and albuterol relaxed constricted airway smooth muscle within 3 min. Even 3 mg/kg MIDD0301 was sufficient to reduce AHR during the first and second doses after the methacholine challenge. Taken together, these data support the use of MIDD0301 as a fast-acting bronchospasm reliever that quickly reverses bronchoconstriction induced by methacholine and retains effectiveness upon repeated nebulized administrations.

Steroid resistant asthma is often associated with elevated levels of IFN γ and is consistent with a neutrophilic rather than eosinophilic disease endotype.²⁶ The underlying pathogenic mechanism could be microbial lung infection leading to asthma, which is controlled poorly with steroids due to impaired nuclear translocation of the liganded glucocorticoid receptor in pulmonary macrophages.²⁷ Intratracheal installation of IFN γ and bacterial lipopolysaccharide (LPS) was used to model microbial infection and induced severe, acute lung inflammation, which was less responsive to oral steroid treatment.²⁸ We thus evaluated the pharmacological effect of nebulized and oral MIDD0301 in the IFN γ /LPS lung inflammation model in comparison to albuterol and two steroids, inhaled fluticasone and oral dexamethasone. The results are summarized in Figure 4.

Twenty-four h after intratracheal installation of LPS and IFN γ , Swiss Webster mice were treated with graded doses of methacholine, resulting in a sRaw of 6.0 cmH₂O*s after the third administration. Our adaptation of the model reported by Li et al.²⁸ employs female Swiss Webster instead of BALB/c mice, which enabled us to use lower doses of nebulized methacholine and spontaneously breathing animals instead of the forced oscillation technique. Nebulization of 7.2 mg/kg albuterol significantly reduced AHR at all methacholine challenges (Figure 4A). Three different doses of nebulized MIDD0301 were administered. At the higher 10 and 7.2 mg/kg MIDD0301 doses, reduction of sRaw was observed at the second and third methacholine challenge. The 3 mg/kg MIDD0301 dose was ineffective at all three methacholine challenges. Thus, for severe LPS- and IFN γ -induced lung inflammation, nebulized MIDD0301 and albuterol demonstrated similar reduction of AHR.

Next, we compared MIDD0301 with the corticosteroid fluticasone using repeated nebulized administration for 3 days (Figure 4B). The recommended human daily dose of fluticasone ranges between 176 and 440 μ g for adults (70 kg), corresponding to 2.5–6.2 μ g/kg. Fluticasone at 10 μ g/kg did not reduce sRaw in the IFN γ /LPS model. To observe a fluticasone effect in this model, we raised the test dose 5-fold

to 50 μ g/kg, which was able to significantly reduce AHR after repeated nebulized administrations at the third and fourth methacholine challenges. It was reported that similar high doses were needed to reduce AHR in mice infected with *Mycoplasma pneumoniae*.²⁹ By comparison, MIDD0301 reduced AHR after repeated doses of 3 mg/kg at the fourth and fifth methacholine challenges. A single nebulization of MIDD0301 just before the methacholine challenge did not reduce AHR (Figure 4A), which reflects an inadequate time to develop pronounced anti-inflammatory effects from repeated daily dosing with MIDD0301. A nebulized repeated dose of 1 mg/kg MIDD0301, on the other hand, was not adequate to improve the LPS/IFN γ -induced lung inflammation.

The report by Li et al.²⁸ stated that orally administered dexamethasone did not reduce AHR in the LPS/IFN γ lung inflammation model, which prompted us to investigate the efficacy of orally administered MIDD0301. For this study, we used female BALB/c mice, which exhibited a different range of sRaw values in response to methacholine than female Swiss Webster mice. Starting with 1.6 mg/mL methacholine and increasing the challenge to 12.5 mg/mL resulted in a sRaw change from 1.4 to 3.8 cmH₂O*s (Figure 4C). Consistent with the work of others, oral dexamethasone at 1 mg/kg/d for 3 days did not reduce methacholine-induced AHR. Higher glucocorticoid doses were avoided because of observed toxicity of orally administered prednisone in our previous study.²⁰ In contrast, 200 mg/kg/d MIDD0301 resulted in lower sRaw values at the third and fourth methacholine challenge. Thus, orally administered MIDD0301 significantly reduced AHR resulting from severe lung inflammation induced by LPS and IFN γ .

To correlate the nebulized exposure of MIDD0301 using a NAM chamber to the amount of MIDD0301 that reaches the lungs, we conducted a pharmacokinetic study to quantify tissue concentrations over 2 h. The results are summarized in Figure 5.

The highest lung concentration of MIDD0301 can be assumed at $t = 0$ min, and the earliest practical time point for measurement was $t = 5$ min. Additional dosed mice were sacrificed at the later indicated time points for tissue harvest. Exponential one-phase nonlinear regression reflected a lung

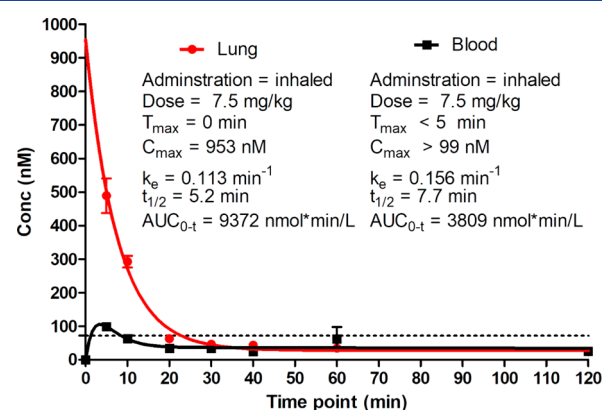


Figure 5. Pharmacokinetics of nebulized MIDD0301 in Swiss Webster mice. Groups of four mice were dosed with 20 μ L of nebulized MIDD0301 (7.5 mM in water pH 7.5). Tissues were harvested at indicated time periods and MIDD0301 quantified by LCMS/MS. Data are depicted as means $\pm$ SD. Data were analyzed with PK Solution 2.0.

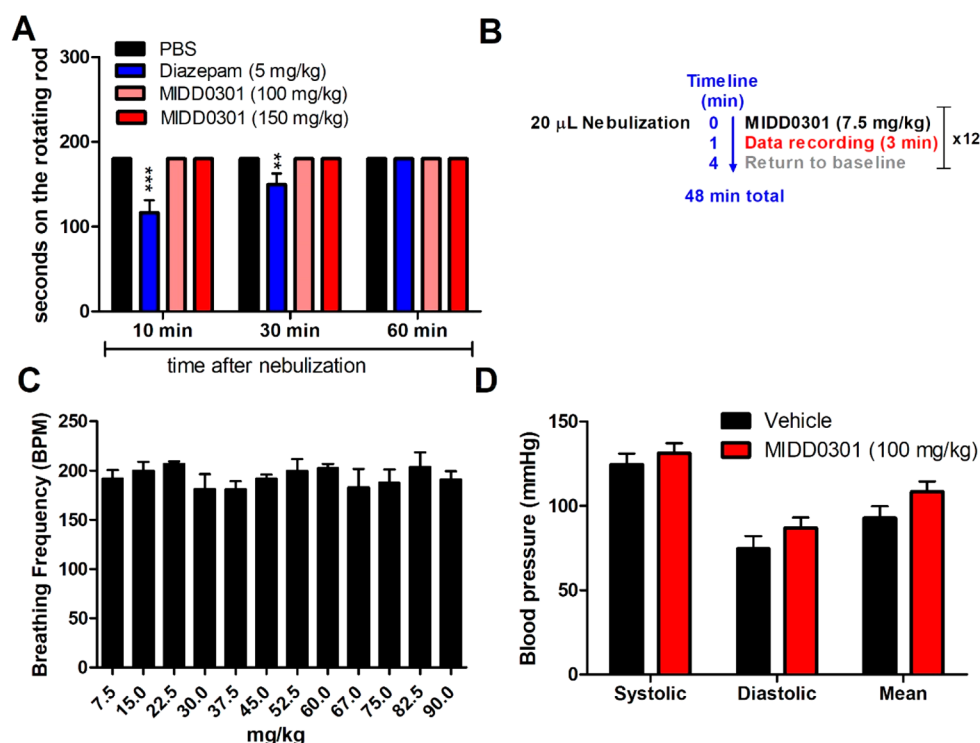


Figure 6. Safety evaluation of nebulized MIDD0301 in mice. (A) Sensorimotor coordination: Compounds were formulated as described in the Materials and Methods section and nebulized at indicated doses using the NAM instrument. After removal from the nebulization chamber, female Swiss Webster mice were tested on a rotarod at 15 rpm for 3 min at 10, 30, and 60 min. The time of fall was recorded if occurring prior to 3 min. Data are expressed as means $\pm$ SEM ($n = 10$). (B) Protocol to determine the respiratory safety of repeated nebulized MIDD0301 dosing using the NAM instrument. (C) Four C57BL/6 mice were subjected to the protocol described in part B and breathing frequencies measured over 3 min after each MIDD0301 nebulization. Data are depicted as means $\pm$ SD. (D) Systolic and diastolic blood pressures were measured with a noninvasive tail-cuff plethysmograph 1 h after oral MIDD0301 administration. Ten measurements were performed for each female Swiss Webster mouse in groups of four. ** ($p < 0.01$) and *** ($p < 0.001$) values were determined by two-way ANOVA.

concentration of 953 nM at $t = 0$; thus, 0.06% of nebulized MIDD0301 was found in the lung assuming the weight of lung and mouse to be 300 mg and 30 g, respectively. The half-life of MIDD0301 in the lung was 5.2 min. MIDD0301 has a binding affinity of 72 nM for the GABA_AR; thus, therapeutic concentrations for receptor activation were maintained at this level for at least 25 min. In addition, MIDD0301 blood concentration and to a lesser degree its lung concentration increased at the 60 min time point, suggestive of intestinal absorption of ingested drug. As pointed out in the Introduction, about 80–90% of inhaled medication is likely to be swallowed.¹⁵ In contrast to the lung, the concentration of MIDD0301 in blood increased marginally, indicating intestinal uptake and metabolism before entering the blood circulation. Similar ratios between lung and blood concentrations were observed for nebulized MIDD0301 administration using a flexiVent respiratory system, with a reported brain concentration of less than 3% of the lung concentration.¹⁰

To confirm minimal brain distribution of MIDD0301 with the absence of central effects, we studied mice that received nebulized MIDD0301 for possible sensorimotor impairment using the rotarod (Figure 6A). Female Swiss Webster mice were trained to balance on a rotating rod for 3 min and tested 10, 30, and 60 min after nebulization of MIDD0301 or positive control compound diazepam, a GABA_AR ligand that is known to achieve high brain concentrations. The lack of central activity, specifically in the medulla, was demonstrated with measurements of breathing frequencies after repeated nebulization with MIDD0301 (Figure 6C). Cardiovascular safety

was investigated with blood pressure measurements after oral administration of MIDD0301 (Figure 6D).

MIDD0301, at nebulized doses of 100 and 150 mg/kg, did not cause sensorimotor impairment (Figure 6A). In contrast, nebulized diazepam at 5 mg/kg exhibited significant CNS effects. These results are in agreement with studies performed for orally administered MIDD0301 and diazepam.⁶ Interestingly, a recent clinical study with inhaled diazepam confirmed bronchodilation for patients with asthma.³⁰ The authors proposed that diazepam acted on the peripheral nervous system regulating the airway smooth-muscle tone rather than directly relaxing contracted airway smooth muscle, which was demonstrated for MIDD0301 using *ex vivo* human lung tissue.¹⁰ To show that bronchodilation by MIDD0301 is limited to constricted airways (e.g., in the presence of methacholine or caused by lung inflammation), we iteratively treated naive mice with nebulized MIDD0301 followed by airway measurements using a whole-body plethysmograph (Figure 6C). Breathing frequencies did not change over a period of 48 min, with a combined dose of 90 mg/kg. Cardiovascular safety of MIDD0301 was shown *in vivo* at a dose of 100 mg/kg using a noninvasive tail-cuff plethysmograph (Figure 6D). Blood pressure values obtained for MIDD0301 were not statistically different from vehicle controls. These results are consistent with earlier published findings on the inability of MIDD0301 to interact with the Kv11.1 potassium ion channel (hERG).²⁰

It can be concluded that MIDD0301 is a potent bronchodilator that is effective in relieving methacholine-

induced AHR in diverse animal models. The therapeutic value of nebulized and orally administered MIDD0301 was demonstrated for lung inflammation induced by allergens and infectious mediators, which correspond to major disease pathways underlying human bronchoconstrictive disorders including asthma. MIDD0301 represents a first-in-class drug based on a fundamentally novel asthma mechanism of action for the treatment of inflammatory respiratory diseases, namely, targeting GABA_AR in the lung. This approach avoids safety and efficacy liabilities of existing drugs, and unlike current treatments, it is effective in reducing airway constriction and lung inflammation when administered by oral and inhaled routes.

MATERIALS AND METHODS

Chemicals. MIDD0301 was synthesized using a published procedure.³¹ Purity of >99% was confirmed by HPLC. Identity was determined by ¹H NMR, ¹³C NMR, and high-resolution mass spectrometry. Albuterol, montelukast, dexamethasone, fluticasone, and diazepam were purchased from MilliporeSigma (St. Louis, MO) and used without further purification.

Drug Formulation. For nebulization, a 3 mg/mL solution of MIDD0301 was prepared in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) or water and pH adjusted to 7.2 with NaOH. For other compound concentrations, this solution was diluted with water. For oral administration, MIDD0301, montelukast, and dexamethasone were suspended in polyethylene glycol (MilliporeSigma, St. Louis, MO) and diluted in a 2% aqueous solution of hydroxypropyl methylcellulose (MilliporeSigma, St. Louis, MO). The final polyethylene glycol concentration was 2.5%. Albuterol was dissolved in water for nebulized administration. Fluticasone and diazepam were dissolved in PBS with 0.17% Tween-80.

Experimental Animals. Male and female BALB/cJ, female A/J, and male C57/BL6J were purchased from Jackson Laboratory (Bar Harbor, ME), and female Swiss Webster mice were purchased from Charles River Laboratory (Wilmington, MA). Female C57/BL6-129S6 hybrid mice were obtained from a breeding colony at the University of Wisconsin-Milwaukee. The mice were offspring from C57BL/6J female and *Scn1a*[±] (37107-JAX, 129S6 background) male and tested negative for this mutation. Animals were housed in a pathogen-free and 12 h light and dark cycle environment. Animals had *ad libitum* access to food and water. UW-Milwaukee and Columbia University confirmed that all animal experiments were in compliance with their Institutional Animal Care and Use Committees.

Measurement of sRaw Using the Buxco FinePointe Noninvasive Airway Mechanics Instrument (DSI, St. Paul, MN). Mice were trained once a day for 5 days to become accustomed to the measuring chambers during nebulization and data acquisition. Instrument calibration was carried out before each experiment. Each study protocol is described in the **Results and Discussion**. Specific airway resistance (sRaw) was computed with FinePoint software using ventilation parameters recorded for the nasal and thoracic chambers. Compounds were nebulized as indicated for each experiment. Methacholine was dissolved in PBS and nebulized as indicated for each experiment. Nebulizers were calibrated for each measurement. Usually, nebulization occurred for 1 min followed by a 3 min data acquisition and a 1 min pause before the next methacholine nebulization. Data analysis was carried out

with GraphPad Prism (GraphPad, San Diego, CA) using two-way ANOVA and Bonferroni posttest.

Ovalbumin (OVA) Sensitization and Challenge. Male C57BL/6 or male BALB/cJ mice were sensitized with intraperitoneal (i.p.) injections of 100 μ L of a 0.9% NaCl aqueous solution with 0.5 mg/mL OVA (Sigma-Aldrich, St. Louis, MO) and 20 mg/mL alum (Imject Alum; 40 mg/mL, Thermo Scientific, Pierce, Rockford, IL) on days 0, 7, and 14. During days 22–27, mice were anesthetized with isoflurane and challenged daily with 1 mg/kg OVA intranasally. AHR measurements were conducted on day 28.

House Dust Mite (HDM) Sensitization. Eight-week-old male C57BL/6 mice were briefly anesthetized with isoflurane (Baxter, Deerfield, IL) and administered HDM antigen (30 μ g dissolved in 25 μ L of PBS; Greer Laboratories, Lenoir, NC) or PBS alone (nonsensitized controls) intranasally while spontaneously breathing once a day for 3 weeks.

Measurement of Resistance (Rn) Using flexiVent (Scireq, Montreal, QC, Canada). Mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.), paralyzed with succinylcholine (10 mg/kg i.p.), and mechanically ventilated via a 20 g cannula inserted into the trachea via a tracheostomy (tidal volume, 10 mg/kg; 150 breaths/min; positive end-expiratory pressure, 3 mmHg). Some mice received nebulized MIDD0301 (5 mM in PBS) (10 s nebulization, 50% duty cycle; $\sim$ 18.3 μ L delivered)³² or vehicle (PBS) 10 min before the measurement of the central lung resistances (Rn) by the forced oscillatory technique during a graded, nebulized methacholine challenge (0–50 mg/mL). Electrocardiography and temperature monitoring were performed throughout the experiment. Resistance values for each mouse at each methacholine dose represent an average of three measurements. Data analysis was carried out with GraphPad Prism (GraphPad, San Diego, CA) using two-way ANOVA and Bonferroni posttest.

LPS/INF γ Challenge. Eight-week-old female Swiss Webster or female BALB/c mice were anesthetized with a 100 μ L i.p. injection of xylazine (2 mg/mL) and ketamine (10 mg/mL). Portions of 50 μ L of a 0.1% bovine serum albumin solution containing 30 μ g/mL mouse INF γ (R&D systems, Minneapolis, MN) and 1 μ g of LPS (MilliporeSigma, St. Louis, MO) were administered intratracheally. AHR was determined 24 h after installation.

Pharmacokinetic Study. Ten-week-old female Swiss Webster mice were administered nebulized MIDD0301 at 7.5 mg/kg in PBS using a Buxco NAM chamber (DSI, St. Paul, MN). At indicated time points, groups of four animals were sacrificed and blood collected via cardiac puncture into heparinized tubes. Lungs were harvested, placed on dry ice, and later transferred to a -80°C freezer. Blood samples were thawed on ice, vortexed for 10 s, and 200 μ L combined with 400 μ L of cold methanol containing 300 nM XHE-III-74A⁹ as internal standard (I.S.). Samples were vortexed for 30 s and centrifuged at 7,000g for 10 min at 4°C . The supernatant layer was then transferred into a new tube and evaporated using a Speedvac concentrator (Thermo Fisher Scientific, Waltham, MA). The residue was reconstituted with 200 μ L of methanol and spin-filtered through a 0.22 μ m nylon centrifugal filter unit (Costar). For the analysis, 100 μ L of each filtrate was combined with 10 μ L of a 500 nM methanol solution of SH-053-2F'-R-acid⁷ as second internal standard. Lung tissue samples were thawed, weighed, and homogenized directly with 500 μ L of methanol, containing 300 nM XHE-III-74A using a

Cole Palmer LabGen 7B Homogenizer. Samples were centrifuged for 10 min at 7,000g at 4 °C. The supernatant was then spin-filtered (0.22 μ m), evaporated, and reconstituted as described for the blood samples. Ultrahigh performance liquid chromatography was performed with Shimadzu Nexera X2 LC30AD series pumps (Shimadzu, Kyoto, Japan). The injection volume was 5 μ L (LC–MS/MS, Shimadzu 8040). Analytes were separated with an Agilent RRHD Extend-C18 (2.1 mm $\times$ 50 mm, 1.8 μ m particle size) column with gradient elution at a flow rate of 0.5 mL/min. The mobile phase was methanol and water (both containing 0.1% formic acid). Time program: 20% B (0 min) $\rightarrow$ 45% B (2 min) $\rightarrow$ 99% B (4 min), hold at 99% B (4.75 min), return to 20% B (5 min), hold at 20% B (1.5 min). Column Temperature: ambient. Analytes were monitored under positive mode by electrospray and atmospheric pressure ionization run in dual (DUI) mode. The following transitions were monitored in multiple reaction monitoring (MRM) mode. Ion pairs were the following: for MIDD0301, m/z 415.95 > 305.00, m/z 415.95 > 398.00, m/z 415.90 > 357.0, and m/z 415.90 > 329.00, XHE-III-74A: m/z 314.10 > 368.10, m/z 314.10 > 278.10, and m/z 314.10 > 296.15; for SH-053-2F'-R-acid, m/z 314.10 > 296.15, m/z 314 > 278.10, and m/z 314.10 > 268.10. The mass spectrometer was operated with the heat block at 400 °C, drying gas flow at 15 L/min, desolvation line temperature of 250 °C, nebulizing gas flow at 1.5 L/min, and both needle and interface voltages at 4.5 kV. The response acquisition was performed using LabSolutions software. Standard curves were fitted by linear regression and used for quantification. The mean and standard deviation were calculated and data fitted with PK Solution 2.0 to determine PK parameters.

Rotarod. Groups of 10 female Swiss Webster mice were trained to maintain balance at a constant speed of 15 rpm on the rotarod apparatus (Omnitech Electronics Inc., Nova Scotia, Canada) for 3 min. MIDD0301 in PBS (3.2 mg/mL) was administered as an aerosol using a NAM chamber (DSI, St. Paul, MN). After 10, 30, and 60 min after each nebulization, mice were placed on the rotarod for 3 min. If a mouse fell before the 3 min was completed, it was placed again on the rod. If a mouse fell for the second time, the time of the fall was recorded. Data analysis was carried out with GraphPad Prism (GraphPad, San Diego, CA) using two-way ANOVA and Bonferroni posttest.

Blood Pressure Measurements. Mice were trained for 3 days before the measurement to stay calm in the heating chamber for 10 min with continuous blood pressure measurements. The measurements were performed at the same time each day in absolute silence, with no extraneous odors, and with the least possible handling. Systolic and diastolic blood pressures were measured by tail-cuff plethysmography (IITC Life Science Inc., Woodland Hills, CA). The heat chamber was set at 28–30 °C for optimal tail arterial dilation to allow the measurement of the pulsatile pressure. A tail cuff/sensor was inflated by the system to a maximum pressure of $\sim$ 250 mm/Hg, and systolic blood pressure and pulse were determined using the optical sensor. Animals were administered vehicle or MIDD0301 by oral gavage, and measurement occurred 1 h after administration. Ten repeated measurements were performed with each mouse with a group size of four. Data analysis was carried out with GraphPad Prism (GraphPad, San Diego, CA) using two-way ANOVA and Bonferroni posttest.

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Notes

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■ ABBREVIATIONS

ASM, airway smooth muscle; GABA_AR, gamma amino butyric acid type A receptor; IL-17A, interleukin 17A; IL-4, interleukin 4; IL-10, interleukin 10; TNF α , tumor necrosis factor alpha; CNS, central nervous system; BAA, β -adrenergic receptor agonist; COPD, chronic obstructive pulmonary disease; NAM, noninvasive airway mechanics instrument; SEM, standard error of the mean; OVA, ovalbumin; HDM, house dust mite; Rn, airway resistance; sRaw, specific airway resistance; IFN γ , interferon gamma; LPS, bacterial lipopolysaccharide; PBS, phosphate-buffered saline, hERG, human ether-à-go-go, DUIS, dual-ionization source; MRM, multiple reaction monitoring; NMR, nuclear magnetic resonance; LCMS/MS, liquid chromatography-tandem mass spectrometry.

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