MIDD0301 – A First-in-class Anti-inflammatory Asthma Drug Targets GABA\(_A\) Receptors Without Causing Systemic Immune Suppression

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Abstract

We report a 28-day repeat dose immunotoxicity evaluation of investigational drug MIDD0301, a novel oral asthma drug candidate that targets gamma amino butyric acid type A receptors (GABA\(_A\)R) in the lung. The study design employed voluntary oral administration of mice twice daily throughout the study period with 100 mg/kg MIDD0301 mixed in peanut butter. Compound dosing did not reveal signs of general toxicity as determined by animal weight, organ weight, or hematology. Peanut butter plus test drug (in addition to ad libitum standard rodent chow) did not affect weight gain in the adult mice, in contrast to weight loss in 5 mg/kg prednisone-treated mice. Spleen and thymus weights were unchanged in MIDD0301-treated mice, but prednisone significantly reduced the weight of those organs over the 28-day dosing. Similarly, no differences in spleen or thymus histology were observed following MIDD0301 treatment, but prednisone treatment induced morphological changes in the spleen. The number of small intestine Peyer’s patches was not affected by MIDD0301 treatment, an important factor for orally administered drugs. Circulating lymphocyte, monocyte, and granulocyte numbers were unchanged in the MIDD0301-treated animals, whereas differential lymphocyte numbers were reduced in prednisone-treated animals. MIDD0301 treatment did not alter IgG antibody responses to DNP following DNP-KLH immunization, indicating that systemic humoral immune function was not affected. Taken together, these studies show that repeated daily administration of MIDD0301 is safe and not associated with adverse immunotoxicological effects.

Keywords: GABA\(_A\)R, asthma, immunotoxicity, peanut butter, MIDD0301
Introduction

Asthma is an increasing public health challenge with a patient population approaching 10% of the global population. Low-dose inhaled corticosteroid (with or without a long-acting β2-agonist) inhalers are the mainstays of asthma treatment. Yet, in refractory asthma, disease responds poorly to corticosteroids, prompting increasing oral dosing that is linked to greater side effects and ineffective in steroid-tolerant disease. Injectable biologics can effectively target inflammation in a subset of asthma patients, but high costs permit their use in only severe disease. In contrast to these therapies, MIDD0301 has been developed to target GABA₅R on airway smooth muscle (ASM) and inflammatory cells, providing a novel asthma drug mechanism of action.

MIDD0301 is positive GABA₅R modulator that has been shown to alleviate multiple symptoms of asthma in animal models after oral dosing. The GABA₅R is a membrane chloride ion channel that opens in the response to GABA. The receptor is a heteropentamer (comprised of subunits: α1–6, β1–3, γ1–3, δ, ε, π, θ, ρ1-3), and most often takes the form of two alpha, two beta, and one tertiary subunit. During the last decades, the role of GABA₅Rs in the brain has been studied extensively and several drugs targeting this receptor are currently available to treat a variety of CNS indications. Recently, GABA₅R subunits have been identified in many non-CNS cells (human protein atlas). Importantly, GABA₅R alpha subunits have been identified in several cell types associated with asthma such as airway epithelia cells, ASM, and airway leukocytes (such as CD4⁺ T cells and alveolar macrophages). MIDD0301 relaxes constricted ASM and reduces airway hyperresponsiveness in an ovalbumin induced murine model of asthma. MIDD0301 has a half-life of almost 4 hours in the lung and extremely low brain distribution. In sensorimotor studies, no CNS effects were observed at 1000 mg/kg. Importantly, MIDD0301 has excellent anti-inflammatory properties, reducing eosinophil and macrophage numbers in the lungs of asthmatic mice. A change of CD4⁺ T cell transmembrane current was observed in the presence of MIDD0301 (EC₅₀ = 17 nM) and 20 mg/kg MIDD0301 p.o. was sufficient to reduce CD4⁺ T cell numbers in the asthmatic mouse lung. Furthermore, reduction of specific pro-inflammatory cytokines, such as IL-17, IL-4, and TNFα, was observed in MIDD0301 treated asthmatic mice, confirming the immunoregulatory properties of MIDD0301.

International regulatory bodies require that investigational new drugs be evaluated for the potential to produce immunosuppression (e.g., FDA Guidance, Oct. 2002 and ICH Expert Working Group advises to carry out immunotoxicity studies in accordance with the European Union, Japan and US). These studies are especially important for investigational drugs that are designed to reduce immune responsiveness or suspected to have immune suppression as a side effect. The importance of this study is the first systematic evaluation of a drug specifically targeting the GABA₅R on immune cells, showing that therapeutically relevant anti-inflammatory activity can be achieved without off-target systemic immune suppression. Here, in addition to anatomical and histological measures, we adopted ICH S8 guidelines to investigate systemic immune suppressive effects of MIDD0301 by quantifying T cell dependent humoral immune responses to DNP following immunization with DNP-keyhole limpet hemocyanin (DNP-KLH). Safety of MIDD0301 was initially shown by administering twice daily doses of up to 100 mg/kg in mice. No adverse side effects were observed during five days of this treatment. Herein, we expand upon these studies, and report the evaluation of the pharmacological effects of MIDD0301 in a 28-day repeat dose study with 100 mg/kg b.i.d. MIDD0301. A battery of anatomic, hematologic, and immunologic measures were evaluated in male and female mice to uncover any immune related toxicities.
Results

Previous oral dosing of MIDD0301 in mice for pharmacodynamic studies was carried out by oral gavage using 2% hydroxypropylmethylcellulose and 2.5% polyethylene glycol. During studies of five-day duration, no significant average animal weight changes were observed. We then carried out a pilot study wherein a small group of mice were gavaged for seven days b.i.d. with vehicle alone (Figure 1) to determine if this route would be suitable for longer-term studies. This study showed that all mice receiving b.i.d. vehicle gavage lost weight during the seven-day trial. Oral gavage is a very accurate route of administration, however, it has been reported that even when carried out by very experienced personnel, respiratory complications, stomach distension, and inflammation due to small laceration of the esophagus can occur. Furthermore, it has been shown that over time gavaged mice will show impaired weight gain due to reduced food intake. Handling and restraining of animals during the feeding further induces stress, which has been associated with decreased food intake.

![Figure 1. Weight changes of individual mice that were fed twice a day with 2% hydroxypropylmethylcellulose and 2.5% polyethylene glycol using oral gavage.](image)

Based on these factors, we chose a voluntary oral administration regimen, which has been reported by others where glucose jelly was used to deliver the anti-obesity drug Rimonabant. However, we decided to use peanut butter because of better uniformity for the formulation. To initiate the study, two groups of mice were trained to consume 100 mg of peanut butter twice a day for one week. To ensure precise administration of peanut butter or peanut butter with 100 mg/kg MIDD0301, each mouse was placed singly in a small feeding container with the measured amount of peanut butter and returned to the group housing cage after consumption. A third group of mice was not treated nor moved into feeding containers. The body weights of mice over the course of the study are shown in Figure 2.

![Female body weight](image)

![Male body weight](image)
Figure 2. Average mouse body weights over the 28-day study. In separate feeding containers, Swiss Webster male and female mice (8 weeks of age) were voluntarily administrated peanut butter or peanut butter + 100 mg/kg MIDD0301 b.i.d. Mice in the “no treatment” group were not moved into feeding containers. Animals were weighed every week (mean ± SD, n = 5). ANOVA was used for statistical analysis. Significant differences (p≤0.05) between groups were not observed at any of the time points.

The average starting weight of the 8 week old female Swiss Webster mice was lower than the corresponding males. During the course of the study, the average weights of both MIDD0301 treated adult female and male mice did not change significantly compared to control groups (peanut butter alone or no treatment). The average Swiss Webster female weight after 12 weeks was 26 ± 2 grams and 35 ± 2 grams for males, which is in agreement with the average expected weight for this mouse strain. In addition to weight measurements, animals were observed for any overt signs of gastrointestinal distress, grooming changes, or other behavior abnormalities, of which there were none in any group.

To determine the effect of MIDD0301 on the systemic immune function, female and male 6 week old Swiss Webster mice were immunized with 50 µg DNP-KLH in alum on day one and day 21. Volunteered administration of peanut butter formulated MIDD0301 (100 mg/kg, b.i.d.), peanut butter + prednisone (5 mg/kg/day), or peanut butter alone was carried out for 28 consecutive days. During that period, mice were weighted on day 14 and 28 (Figure 3).

![Figure 3](image)

Figure 3. Effect of DNP-KLH immunization on average body weight. In separate feeding containers, Swiss Webster female and male mice (6 weeks of age at the beginning of the study) were voluntarily administrated peanut butter, peanut butter + MIDD0301 (100 mg/kg, b.i.d.), or peanut butter + prednisone (5 mg/kg/day). Three groups were immunized with DNP-KLH on day 1 and day 21. Animals were weighed on days (14) and (28). Data are expressed as mean ± SEM, n = 5. ANOVA was used for statistical analysis, *** = P < 0.001.

The average weight of the female mice increased as expected for the non-immunized and DNP-KLH immunized control groups over the course of four weeks. No significant changes were observed between these two groups. Furthermore, no significant changes were observed for the MIDD0301 (100 mg/kg b.i.d.) and prednisone (5 mg/day) treated females mice in comparison to their corresponding peanut butter only control group. Consistent with the results for female mice, DNP-KLH immunization did not cause significant weight changes in male mice over the study duration in comparison to the non-immunized mice. The average weight of male mice administered MIDD0301 (100 mg/kg b.i.d.) was similar to the peanut butter only control group. However, DNP-KLH immunized male mice treated with prednisone (5 mg/kg/day) had a significantly reduced average weight at the 28 day time point in comparison to peanut butter only treated DNP-KLH immunized male mice.
Lymphoid organs and tissues, including thymus, spleen, and Peyer’s patches were evaluated in all mouse groups after the 28 day MIDD0301 or prednisone treatment regimens. The results are presented in Figure 4.

Figure 4. Evaluation of lymphoid organs and Peyer’s patches in DNP-KLH immunized Swiss Webster mice after 28 days of treatment. Mice were 6 weeks old at the beginning of the study. A-C) Female Swiss Webster mice were voluntarily administrated peanut butter, peanut butter + MIDD0301 (100 mg/kg b.i.d.) or peanut butter + prednisone (5 mg/kg/day). D-F) Corresponding groups of male Swiss Webster mice were treated in the same fashion. After 28 days, organs were harvested and weighted and intestines dissected for Peyer’s patch counting. Data are expressed as mean ± SEM, n = 5. ANOVA was used for statistical analysis: * = p < 0.05; ** = p < 0.01, and; *** = p < 0.001.

No significant differences were observed for spleen and thymus weights and Peyer’s patch numbers between peanut butter fed DNP-KLH immunized and non-immunized animals (Figure 4, A-F). Spleen and thymus from DNP-KLH immunized MIDD0301-treated male and female mice had weights similar to corresponding DNP-KLH immunized mice given peanut butter alone. However, both male and female mice treated with 5 mg/kg/day prednisone had significantly smaller spleen and thymus weights in comparison to peanut butter only treated DNP-KLH immunized mice. The numbers of Peyer’s patches were unchanged in all groups. Lymphoid organs were further evaluated for gross histological alterations by H&E staining. The images are shown in Figure 5.
Figure 5. H&E stained sections of mouse spleens (A-H, 40x) and thymus (I-P, 100x). A&I) male mice, peanut butter, B&J) female mice, peanut butter, C&K) male DNP-KLH-immunized mice, peanut butter, D&L) female DNP-KLH-immunized mice, peanut butter, E&M) male DNP-KLH-immunized mice, peanut butter + MIDD0301 (100 mg/kg) b.i.d. for 28 days, F&N) female DNP-KLH-immunized mice, peanut butter + MIDD0301 (100 mg/kg) b.i.d. for 28 days, G&O) male DNP-KLH-immunized mice, peanut butter + prednisone 5 mg/kg/day for 28 days, H&P) female DNP-KLH-immunized mice, peanut butter + prednisone 5 mg/kg/day for 28 days.

H&E stained spleen sections reveal typical histological presence of white (lymphoid follicles) and red pulp in all mouse groups. The cellular arrangements of follicles show no remarkable differences among the groups, except for the prednisone-treated animals (Figure 5, G and H) that exhibited smaller and less uniform follicle size. Similar splenic changes have been reported in patients taking corticosteroids over a long period of time.2 The H&E staining also highlighted the lymphocyte rich white pulp, with well-defined features of centrally located germinal centers and the surrounding marginal zone for all spleen sections. The thymus histology of all groups appeared unchanged following treatment. The medulla and cortex regions were well defined in all groups and the thymus cell density appeared unchanged in all groups. Overall, no significant changes in the spleen and thymus histology were observed after repeated MIDD0301 treatment, however, prednisone-treated animals did exhibit some alteration in spleen histology.

Blood was collected from mice after the 28-day treatment regime for hematologic evaluation. The evaluation is summarized in Table 1.

Table 1. Hematology parameters for non-immunized and DNP-KLH-immunized Swiss Webster mice after indicated treatment for 28 days. The data are expressed as mean ± SD, n = 5.
Overall, no significant differences in hematology parameters were found among the four different treatment groups as determined by ANOVA analysis. However, the differential lymphocyte values trended lower in the prednisone-treated male and female groups, whereas the differential granulocytes were the highest in these groups. An elevation of granulocytes would be consistent with corticosteroid use in humans where granulocyte (particularly neutrophil) levels are known to increase. Importantly, male and female mice treated with MIDD0301 exhibited differential white blood cell counts similar to the control groups, which are in accord with previously reported hematology ranges for adult Swiss Webster mice.

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<table>
<thead>
<tr>
<th>Mouse (Male)</th>
<th>Peanut butter&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Peanut butter&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MIDD0301&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Prednisone&lt;sup&gt;b&lt;/sup&gt;</th>
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<td></td>
<td>WBC x10&lt;sup&gt;3&lt;/sup&gt; (mm&lt;sup&gt;3&lt;/sup&gt;)</td>
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<td></td>
<td>RBC x10&lt;sup&gt;6&lt;/sup&gt; (mm&lt;sup&gt;3&lt;/sup&gt;)</td>
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<td>HGB (g/dl)</td>
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<td>HCT (%)</td>
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<td>MCV (µm&lt;sup&gt;3&lt;/sup&gt;)</td>
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<td>MCHC (g/dl)</td>
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<td>PLT x10&lt;sup&gt;9&lt;/sup&gt; (mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>986 ± 151</td>
<td>880 ± 80</td>
<td>903 ± 82</td>
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<tr>
<td></td>
<td>MPV (µm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>5.4 ± 0.2</td>
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<td>5.3 ± 0.2</td>
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Non-immunized male and female Swiss Webster mice were voluntarily administrated peanut butter (100 mg/day b.i.d); DNP-KLH immunized male and female Swiss Webster mice were voluntarily administrated peanut butter; peanut butter + 100 mg/kg MIDD0301 b.i.d., or peanut butter + 5 mg/kg/day prednisone; white blood cells; red blood cells; hemoglobin; hematocrit; mean corpuscular volume; mean cell hemoglobin; mean cell hemoglobin concentration; red cell distribution width; platelets; mean platelet volume; differential white blood cell count; lymphocytes; monocytes; granulocytes; The data is expressed as mean ± SD, n = 5. ANOVA was used for statistical analysis.
Three groups of female and male mice were immunized with DNP-KLH on day 1 and day 21 of this study. To investigate the effect of MIDD0301 on a T-dependent humoral immune response, serum DNP IgG was measured by ELISA for all mice at day 28 (Figure 6).

As expected, DNP-KLH immunization significantly increased DNP-specific IgG in female and male mice (Figure 6). IgG levels were similar for the DNP-KLH-immunized mice, irrespective of their treatments. Thus, repeat MIDD0301 at 100 mg/kg twice a day did not impair systemic immune function as measured by a secondary antibody response to a classic T-dependent antigen.

Although pharmacokinetic parameters of MIDD0301 have been reported, we further investigated the clearance of MIDD0301 when given chronically by quantifying the average concentration of MIDD0301 in feces and urine. Here, urine and feces from MIDD0301-treated male and female mice were collected throughout the study and quantified by LCMS/MS. The results are summarized in Figure 7.

The average concentration of MIDD0301 in urine was 46.9 mg of MIDD0301 per liter of urine or 113 μM and the concentration of MIDD0301 in feces was 349 mg of MIDD0301 per kilogram of
feces or 841 μmol/kg. The quantification of unchanged MIDD0301 revealed that 75% of MIDD0301 was secreted via feces, whereas 25% was eliminated via urine. The average concentration of MIDD0301 in urine was 46.9 mg/l, thus taking in account the average volume of 1.5 ml urine per day per mouse,$^{24}$ 0.07 mg of MIDD0301 of the 4 mg dose is secreted unchanged via urine. The average concentration of MIDD0301 in feces was 349.0 mg/kg, thus assuming 0.75 g of feces per day per mouse,$^{24}$ 0.26 mg of the 4 mg dose is secreted unchanged via feces. Thus, we can conclude that MIDD0301 undergoes significant metabolism because only 8.2% of MIDD0301 is secreted unchanged.

**Discussion**

Immunotoxicity evaluations are an essential part of preclinical studies to evaluate drug candidates before first in human trials. MIDD0301 is a small molecule developed for the oral treatment of asthma, effective in reducing airway constriction and airway inflammation. In a ovalbumin-induced murine model of asthma, administration of MIDD0301 was able to reduce the number of bronchoalveolar leukocytes that include eosinophils, macrophages, and CD4$^+$ T cells.$^5$ The mode of action of MIDD0301 is novel, targeting GABA$_A$Rs of leukocytes and ASM. It has been shown that resting and activated T cells expressed different subtypes of GABA$_A$Rs; thus selective GABA$_A$R targeting therapeutics can be anti-inflammatory without inducing general suppression of the immune system.

MIDD0301 modulated the transmembrane potential of T cells, which may directly or indirectly reduced the release of intercellular calcium as shown for GABA$_A$R ligand XHE-III-74 acid.$^{26}$ In turn, calcium homeostasis mediates the immune response of T cells.$^{27}$ To probe further the influence of MIDD0301 on systemic immunity, high doses of MIDD0301 (100 mg/kg) were administrated orally twice a day. The differential white cell counts demonstrated no changes in the number and ratio of lymphocytes, monocytes, and granulocytes in the blood of MIDD0301 and vehicle treated animals. Furthermore, lymphoid organs (spleen and thymus) and Peyer’s patches were unchanged by treatment as determined by organ weight and histology. This is an important finding, because oral dosing of other anti-inflammatory agents such as dexamethasone are known to induce severe apoptosis of intestinal lymphatic tissue and reduced the numbers of Peyer’s patches.$^{28}$ Our results are consistent with this observation, where prednisone treatment caused the reduction of spleen and thymus mass for both female and male mice. Female and male mice were immunized with DNP-KLH to determine if MIDD0301 caused systemic immune suppression. However, DNP-specific antibody levels showed that volunteered administration of MIDD0301 twice a day for 28 days did not diminish the T-dependent response.

Pharmacokinetic experiments with orally administered MIDD0301 have shown a high AUC of 84.0 μM/h in serum (t$_{1/2}$ = 13.9 h) and 56.0 μM/h in lung tissue (t$_{1/2}$ = 3.9 h) using a 25 mg/kg dose in mice.$^5$ In addition, we have reported that MIDD0301 is very stable in the presence of human and mouse liver microsomes with half-lives of 25.7 and 9.2 hours, respectively. We reported herein, that the concentration of unchanged MIDD0301 in feces and urine is below 10% of the administered dose, thus it can be hypothesized that phase II metabolism plays an important role for MIDD0301 clearance. A similar low renal excretion has been shown for unchanged NSAID bearing a carboxylic acid function and substantial hepatic conjugation has been reported.$^{29}$ Thus, future studies will investigate the phase II metabolism of MIDD0301.
Taken together, we have demonstrated that MIDD0301 induces no adverse immunotoxicological effects at 100 mg/kg b.i.d. over a period of 28 days. The production of antibodies following DNP-KLH immunization was not diminished by MIDD0301, although in an ovalbumin-induced model of asthma, MIDD0301 was able to reduce the number of leukocytes in the lung. Accordingly, MIDD0301 is an anti-inflammatory agent that selectively reduces inflammation in the lung without compromising systemic immune function. Finally, MIDD0301 is a safer alternative to prednisone, that at a dose of 5 mg/kg/day (2.5% of the MIDD0301 dose) reduced the mass of spleen and thymus, changed the spleen morphology, and reduced animal weight over a period of 28 days.

**Experimental section**

**Chemicals.** MIDD0301 was synthesized using a published procedure. A purity was of >98% was confirmed by HPLC. Identity was determined by ¹H-NMR, ¹³C-NMR, and high resolution mass spectrometry. Prednisone (>98% purity) was purchased from Sigma (P6254-1g). Skippy creamy peanut butter was used as vehicle.

**Experimental animals.** Eight and six week old Swiss Webster male and female mice (Charles River Laboratory) 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 Institutional Animal Care and Use Committee (IACUC).

**MIDD0301 and prednisone formulation and administration.** Oral gavage: 0.2 ml of MIDD0301 in a 2% hydroxypropylmethylcellulose solution (Sigma-Aldrich, St. Louis, MO) and 2.5% polyethylene glycol (Sigma-Aldrich, St. Louis, MO) was administered by oral gavage with 20G gavage needles (Kent Scientific Corporation, Torrington CT) to a group of mice twice a day for 7 days. Voluntary oral administration: MIDD0301 was formulated in 100 mg of peanut butter at a dose of 100 mg/kg. Twice a day, mice were taken from their cages and put in separate boxes that contained 100 mg of peanut butter or 100 mg of compound formulated peanut butter. The animals were left in the container during the feeding and returned 30 minutes later to their group cage. During that time all the peanut butter was consumed.

**Immunogen preparation and administration:** A 1 mg/ml DNP-KLH aqueous solution was prepared from solid DNP-KLH (Sigma, 324121-100 mg) and 7.5 ml of that solution was added to 7.5 ml of a 40 mg/ml Al(OH)₃ solution (Thermo, Imject Alum 77161). 100 μl of this solution (50 μg DNP-KLH and 2 mg Al(OH)₃) was injected i.p. on day 1 and day 21 for immunization.

**Necropsy.** Mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation and blood withdrawn by cardiac puncture. Half of the blood was combined with EDTA for blood cell and platelet analysis. The other half was coagulated at room temperature and centrifuged for 10 minutes at 2,000 rpm for DNP specific IgG ELISA. After gross pathology, spleen and thymus were removed and fixed in 10% neutral buffered formalin overnight at 4°C, followed three washes with water, and storage in 70% ethanol before sectioning. The small intestine was excised and rinsed with a water using a syringe with a blunt needle. The clean intestine was washed with 7%
(vol/vol) acetic acid/PBS solution and rinse inside using a syringe with a blunt needle. After 5 minutes, the Peyer’s Patches turned white and were readily counted by visual examination.

**Hematology**: EDTA-treated blood samples were analyzed with scil Vet ABC™ Hematology Analyzer providing 13 hematology parameters.

**Histology**: General histology was performed by the Wisconsin Children’s Research Institute including drying, embedding, slicing, and H&E staining. Representative sections were visualized by light microscopy at the indicated magnification.

**Quantification of DNP IgG**: DNP IgG was quantified by ELISA using DNP coated wells (Kamiya Biomedical Company #KT-672). Serum from non-immunized mice was diluted 1:10,000 and DNP-KLH-immunized mouse blood was diluted 1:100,000. The assay was performed following the manufacturer’s instructions.

**Quantification of MIDD0301 by LCMS/MS**

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**Abbreviation:** IND; investigational new drug, FDA; US Food and Drug Administration, DNP-KLH; dinitrophenol-keyhole limpet hemocyanin, GABA_A R; GABA_A receptor, AHR; airway hyperresponsiveness, CNS; central nervous system, NMR; nuclear magnetic resonance, HRMS; high resolution mass spectrometry, NSAID; nonsteroidal anti-inflammatory drugs, EDTA; Ethylenediaminetetraacetic acid

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**References**


20. CFW (Swiss Webster mice) strain code: 024 Charles River description.


