

1. Title page.

Pharmacological screening identifies SHK242 and SHK277 as novel arginase inhibitors with efficacy against allergen-induced airway narrowing *in vitro* and *in vivo*.

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2. Running title page.

a) Running title: Novel arginase inhibitors for asthma

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d) List of non-standard abbreviations

ABH, 2(S)-amino-6-borohexanoic acid

AHR, airway hyperresponsiveness

cNOS, constitutive nitric oxide synthase

DAF-2 DA, 4,5-diaminofluorescein

EAR, early asthmatic reaction

EC₅₀, concentration causing 50% of Effect

eNOS, endothelial nitric oxide synthase

IC₅₀, concentration causing 50% inhibition

IgE, Immunoglobulin E

IL, interleukin

iNOS, inducible nitric oxide synthase

LAR, late asthmatic reaction

L-NAME, N(ω)-nitro-L-arginine methyl ester

nNOS, neuronal nitric oxide synthase

NO, nitric oxide

NOS, nitric oxide synthase

OA, ovalbumin

PC₁₀₀, provocation concentration causing 100% increase of pleural pressure

PCLS, precision-cut lung slices

P_{pl}, pleural pressure

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rhArg, recombinant human arginase

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3. Abstract.

Arginase is a potential target for asthma treatment. However, currently there are no arginase inhibitors available for clinical use. Here a novel class of arginase inhibitors was synthesized and their efficacy was pharmacologically evaluated. The reference compound 2(S)-amino-6-borono-hexanoic acid (ABH) and >200 novel arginase inhibitors were tested for their ability to inhibit recombinant human arginase 1 and 2 *in vitro*. The most promising compounds were separated as enantiomers. Enantiomer-pairs SHK242 and SHK243, and SHK277 and SHK278 were tested for functional efficacy by measuring their effect on allergen-induced airway narrowing in lung slices of ovalbumin-sensitized guinea pigs *ex vivo*. A guinea pig model of acute allergic asthma was used to examine the effect of the most efficacious enantiopure arginase inhibitors on allergen-induced airway hyperresponsiveness (AHR), early and late asthmatic reactions (EAR and LAR) and airway inflammation *in vivo*. The novel compounds were efficacious in inhibiting arginase 1 and 2 *in vitro*. The enantiopure SHK242 and SHK277 fully inhibited arginase activity with IC₅₀-values of 3.4 and 10.5 μM for arginase 1, and 2.9 and 4.0 μM for arginase 2, respectively. Treatment of slices with ABH or novel compounds resulted in decreased ovalbumin-induced airway narrowing compared to control, explained by increased local nitric oxide production in the airway. *In vivo*, ABH, SHK242 and SHK277 protected against allergen-induced EAR and LAR, but not against AHR or lung inflammation. We have identified promising novel arginase inhibitors for the potential treatment of allergic asthma that were able to protect against allergen-induced early and late asthmatic reactions.

4. Significance statement.

Arginase is a potential drug target for asthma treatment, but currently there are no arginase inhibitors available for clinical use. We have identified promising novel arginase inhibitors for the potential treatment of allergic asthma that were able to protect against allergen-induced early and late asthmatic reactions. Our new inhibitors show protective effects in reducing airway narrowing in response to allergens and reductions in the early and late asthmatic response.

5. Introduction

Allergic asthma is a common chronic airway disease that affects millions of people worldwide. It is characterized by recurrent airways obstruction, airway hyperresponsiveness (AHR), airway inflammation and airway remodeling. In allergic asthma patients, exposure to inhaled allergens results in an early asthmatic reaction (EAR) driven by allergen-specific immunoglobulin E (IgE) that binds to surface receptors on basophils and mast cells (Liu et al., 1991; Murray et al., 1985). Mast cell activation leads to release of pro-inflammatory mediators, including histamine and leukotrienes, which trigger airway smooth muscle contraction resulting in narrowing of the airway and airflow obstruction (Bousquet et al., 2000; Martin et al., 2000). Furthermore, mast cell mediators induce an inflammatory cascade triggering infiltration of circulating inflammatory cells (mostly eosinophils) to the lung and in most cases the development of a late asthmatic reaction (LAR)(Busse and Lemanske, 2001). The common treatment option for asthma patients consists of a combination of inhaled anti-inflammatory corticosteroids and bronchodilating long-acting β 2-adrenoceptor agonists (Barnes, 2011). More recently, the anti-cholinergic tiotropium, anti-IgE, anti-interleukin (IL)-5/5-receptor and anti-IL-4-receptor have been added to the Global Initiative for Asthma (GINA) guidelines for patients with severe asthma (Reddel et al., 2019). However, for many patients the current treatment options are not sufficient (Swedin et al., 2017). As a result, groups of patients, especially those suffering from severe asthma, do not receive optimal treatment for their illness. Thus, novel therapeutics to treat asthma are urgently awaited. Previous work from our group indicates that the enzyme arginase plays a key regulatory role in allergic asthma suggesting that arginase may represent one such alternative drug target (Maarsingh et al., 2008; Meurs et al., 2019).

Arginase is a metalloenzyme responsible for the catalytic conversion of L-arginine into urea and L-ornithine. Downstream metabolism of L-ornithine results in the formation of L-proline and polyamines, which are involved in collagen formation and cell proliferation, respectively (Wu

and Morris, 1998). The two isoforms of arginase, arginase 1 and arginase 2, are expressed throughout the human body, including in cell types lacking a complete urea cycle, such as endothelial and epithelial cells, fibroblasts, alveolar macrophages and inhibitory nonadrenergic noncholinergic neurons in the lung (Maarsingh et al., 2009; Que et al., 1998). Their expression can be induced by proinflammatory agents such as Th2-cytokines and cigarette smoke (Maarsingh et al., 2009). In the airways there is competition between arginase and NOS for the common substrate L-arginine (Maarsingh et al., 2006; Maarsingh et al., 2009; Meurs et al., 2002). Even though the affinity of NOS for L-arginine is approximately a 1000-fold higher than that of arginase, competition is still possible as the maximal enzymatic rate (V_{max}) of arginase is much higher (~1000-fold) than that of NOS (Wu and Morris, 1998). Arginase activity is increased in experimental models of asthma and this results in a deficiency of bronchodilatory and anti-inflammatory NO, contributing to allergen-induced airway obstruction, inflammation and hyperresponsiveness (Bratt et al., 2010; Maarsingh et al., 2008; North et al., 2009).

In a previous study we found that arginase inhibition using 2(S)-amino-6-boronohexanoic acid (ABH) reduces allergen-induced EAR and LAR, AHR and eosinophilic inflammation in a guinea pig model of allergic asthma (Maarsingh et al., 2008). Moreover, using perfused tracheal preparations of allergen-sensitized and challenged guinea pigs, we showed that arginase inhibition was able to reduce methacholine induced contractions *ex vivo* (Maarsingh et al., 2009; Meurs et al., 2002). In murine models of allergic asthma, systemic treatment with arginase inhibitors leads to an increase in NO metabolite concentrations (Ckless et al., 2008; Kenyon et al., 2008) and reduced AHR to methacholine in central and peripheral airways (North et al., 2009). Importantly, arginase expression is also enhanced in lung biopsies of asthma patients (North et al., 2009; Zimmermann et al., 2003). In addition, there is an association between arginase expression in bronchial brushings, serum arginase activity, plasma L-arginine and metabolite concentrations to disease severity (Morris et al., 2004; Vonk et al., 2010; Xu et

al., 2017). Furthermore, polymorphisms of arginase 1 and arginase 2 are associated with asthma, asthma severity and reduced responsiveness to β_2 -agonists and glucocorticosteroids (Litonjua et al., 2008; Vonk et al., 2010). These findings make arginase an interesting target for treatment of allergic airway diseases.

Currently there are no arginase inhibitors available for clinical use, and current inhibitors, such as 2(S)-amino-6-boronohexanoic acid (ABH) and N^ω-hydroxy-nor-L-arginine (nor-NOHA), show poor pharmacokinetic profiles (Pudlo et al., 2017). The development of novel arginase inhibitors for their therapeutic use in allergic asthma and rhinitis is therefore of interest (Meurs et al., 2019). In this study, we first confirmed the role of arginase in *ex vivo* and *in vivo* guinea pig models of allergic asthma for screening and efficacy analysis of compounds. Novel arginase inhibitors for the potential treatment of allergic asthma were synthesized and their efficacy was pharmacologically evaluated. The novel arginase inhibitors were tested for their ability to inhibit recombinant human arginase (rhArg) 1 and 2 *in vitro* and compared to ABH. For assessing functional efficacy, their effect on allergen-induced airway narrowing in precision-cut lung slices (PCLS) of ovalbumin (OA)-sensitized guinea pigs was measured *ex vivo*. Furthermore, a guinea pig model of acute allergic asthma (Meurs et al., 2006) was used to examine the effect of the most promising novel arginase inhibitors on allergen-induced EAR and LAR, AHR to histamine after both reactions, and airway inflammation *in vivo*.

6. Methods

Arginase inhibitors

>200 novel arginase inhibitors were developed using structure-based design (Anchor QueryTM) and multicomponent reactions as described in (Domling et al., 2012; Koes et al., 2012). Structures and synthesis routes of the inhibitors are listed in EPO 19180160.2. The SHK-compound series consists of >200 unique compounds. SHK053, SHK081-2, SHK081-5,

SHK099, SHK100 and SHK186-6 represent compounds of different structure classes. SHK081-5 and SHK186-6 were separated into the enantiopure compounds SHK277 and SHK278, and SHK242 and SHK243 respectively. All novel arginase inhibitors are part of patent EPO 19180160.2 (patent submitted). The potent experimental arginase inhibitor ABH and a potent structurally related analogue reported previously (Golebiowski et al., 2013) served as reference compounds.

Animals and sensitization

Male Dunkin Hartley guinea pigs (outbred and specified pathogen-free; Envigo, NL) weighing approximately 250 grams at time of sensitization were used for this study. The animals were actively IgE-sensitized to OA by injecting an allergen suspension containing 100 µg/ml OA and 100 mg/ml Al(OH)₃ in saline. Each animal was injected with 1.0 ml of suspension, of which 0.5 ml was injected i.p. and 0.5 ml was divided over seven s.c. sites close to lymph nodes in the neck, paws and lumbar region (Meurs et al., 2006). All protocols described were approved by the University of Groningen Committee for Animal Experimentation (Application number: AVD10500201581, permit date: 4 June 2015). Guinea pigs were housed conventionally in pairs (for the slice studies) or individually in ventilated cages (for lung function measurements in instrumented animals), in rooms maintained at a 12h light/dark cycle and were provided ad libitum access to food and water, and cage enrichment.

In vivo measurement of lung function

One week after sensitization, animals underwent surgery to install a pleural balloon catheter for online measurement of lung function by pleural pressure (P_{pl}) changes under conscious and unrestrained conditions, as described previously (Meurs et al., 2006). In short, inside the thoracic cavity a small fluid-filled latex balloon-catheter was surgically implanted. The free end of the cannula was driven subcutaneously to the neck of the animal, where it was exposed and

attached permanently. The intrapleural balloon-catheter was connected to a pressure transducer (TXX-R, Viggo-Spectramed, Bilthoven, Netherlands) via an external saline-filled cannula and P_{pl} was continuously measured using an online computer system. By combining flow measurements with a pneumotachograph implanted inside the trachea, and pressure measurement with the intrapleural balloon-catheter, it was previously shown that changes in P_{pl} are linearly related to changes in airway resistance and can thus be used as a sensitive index for allergen- and histamine-induced bronchoconstriction. Using this method airway function can be monitored continuously, while the animals are unaware of the measurements being taken (Meurs et al., 2006).

Histamine and allergen provocations

Histamine and OA provocations were performed by inhalation of aerosolized solutions as described previously (Meurs et al., 2006). Provocations were carried out in a specially designed perspex cage of 9 l, which allowed free movement of the guinea pigs. A DeVilbiss nebulizer (type 646) driven by an airflow of 8 l/min provided the aerosol with an output of 0.33 ml/min. The animals were habituated to the experimental procedures as described in (Meurs et al., 2006). An adaptation period of 30 min, followed by a control provocation with saline lasting 3 min, preceded all histamine provocations.

Subsequent provocations with increasing concentration steps of histamine (6.25, 12.5, 25, 50, 75, 100, 125, 150, 175, 200 and 250 $\mu\text{g/ml}$) in saline were performed in order to assess the airway reactivity to histamine. Histamine provocations lasted maximally 3 min and were separated by 8 min intervals. The challenge procedure lasted until P_{pl} was increased more than 100% above baseline. Using linear interpolation of the concentration- P_{pl} curve, the provocation concentration of histamine causing a 100% increase of P_{pl} (PC_{100}) was derived. PC_{100} -values

were used as an index for airway reactivity towards histamine. Within 15 min after the last histamine provocation, P_{pl} returned to baseline.

Allergen provocations were performed by inhalation of 0.05% OA in saline. OA inhalation was discontinued when the first signs of respiratory distress were observed and an increase in P_{pl} of more than 100% was reached. When this did not occur within 3 minutes, a 0.1% OA solution in saline was used subsequently. These methods were described previously in (Meurs et al., 2006).

Experimental protocol

Basal airway responsiveness was measured 24h before OA challenge. Half an hour before OA challenge, the animals were treated via inhalation with the arginase inhibitors ABH, SHK242 or SHK277 (25mM nebulizer concentration, 3 min) or saline as a control. Animals were challenged with OA (0.05-0.1%) until obstruction. 6h (after the EAR) and 24h (after the LAR) after challenge AHR to histamine was determined (Meurs et al., 2006). 25 h after allergen challenge a bronchial alveolar lavage was performed. Allergen-induced AHR was calculated as a ratio of histamine responsiveness pre and post OA-challenge. The magnitudes of the EAR and LAR were determined by measurement of the area under the P_{pl} -time curve between 0-6h and 6-24h, respectively.

Bronchial alveolar lavage

Animals were anaesthetized with isoflurane. The trachea was exposed and cannulated, and the lungs were lavaged. First 5 ml of sterile saline at 37°C was gently injected and recovered from the lungs, and placed on ice, followed by three subsequent aliquots of 8 ml of saline. The lavage samples were centrifuged at 200 g for 10 min at 4°C. The pellets were combined and resuspended to a final volume of 1.0 ml in phosphate-buffered saline (PBS) and total cell numbers were counted manually. Cytospin-preparations were stained with May-Grünwald and

Giemsa stain for cytological examination. A cell differential was performed by counting at least 400 cells in duplicate in a blinded fashion, as described previously (Meurs et al., 2006).

Precision-cut lung slices (PCLS)

PCLS were prepared as described previously (Maarsingh et al., 2019; Ressmeyer et al., 2006) four to ten weeks after sensitization. Medium containing CaCl₂ (1.8mM), MgSO₄ (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), NaH₂PO₄ (1.2 mM), glucose (16.7 mM), NaHCO₃ (26.1 mM), Hepes (25.2 mM) and pen/strep (5000 U/ml) (pH = 7.2) was prepared. Animals were euthanized by an i.c. injection of an overdose of pentobarbital (Euthasol 20%, Produlab Pharma, Raamsdonksveer, the Netherlands) and exsanguinated. In order to fill the lungs, the trachea was cannulated. A low melting agarose (Gerbu Biotechnik GmbH, Wieblingen, Germany) solution (1.5% final concentration, 37°C) containing isoprenaline (1 µM) in medium supplemented with sodium pyruvate (0.5 mM), glutamine (1 mM), MEM-amino acids mixture (1:50), and MEM-vitamins mixture (1:100) was injected into the lungs. Ice was placed on the lungs for at least 30 min to solidify the agarose after which the lungs were removed and placed on ice. The lobes were separated in order to prepare tissue cores with a diameter of 15 mm. Using a tissue slicer (Compressstome™ VF- 300 microtome, Precisionary Instruments, San Jose CA, USA), the tissue cores were sliced in ice-cold medium containing isoproterenol (1 µM) to a thickness of 500 µm (airway narrowing studies) or 250 µm (NO detection). The PCLS were incubated in a humid atmosphere under 5% CO₂/95% air at 37°C and washed every 30 min, 3 times with medium supplemented with sodium pyruvate (0.5 mM), glutamine (1 mM), MEM-amino acids mixture (1:50), and MEM-vitamins mixture (1:100) containing isoprenaline and once with supplemented medium only (no isoprenaline) and kept overnight.

Ex vivo airway narrowing studies

PCLS were used for OA-induced airway narrowing studies. PCLS were placed in wells containing 1 ml supplemented medium at 37°C. A teflon ring was used to fix the slices in position. PCLS were pretreated with arginase inhibitors (0.01, 0.1, 1, 10, 100 µM) and/or the NOS inhibitor N(ω)-nitro-L-arginine methyl ester (L-NAME; 0.1 mM) for 30 minutes or left untreated as a control. OA (0.00001-100 µg/ml) dose-response curves were subsequently established. Using video-assisted microscopy (Eclipse, TS100; Nikon), time-lapse images (1 frame per 2 s) of the PCLS were captured. Using image acquisition software (NIS-elements; Nikon), the airway luminal area was quantified and expressed as percentage of basal area, as described previously (Maarsingh et al., 2019). Furthermore, maximal airway closure (E_{max}) and sensitivity to OA (EC_{50}) were calculated.

Histamine and cysteinyl leukotriene (CLT) determination

Untreated and ABH (1, 10 or 100 µM) pretreated PCLS were challenged with OA (0.1, 1, 10, 100 µg/ml) for 5 min to determine the effect of arginase inhibition on allergen-induced histamine release. Untreated, unchallenged PCLS were used as a control for determination of spontaneous histamine release. Slices were transferred to ice-cold acetic acid (0.08 M), homogenized by sonication (10 s; 60 pulses; Vibra Cell; Sonics, Newton, USA) and centrifuged for 30 min at 15,000 rpm and 4°C.

Histamine levels in supernatant and in homogenized PCLS supernatant were assessed by liquid chromatography in combination with isotope dilution tandem mass spectrometry (LC-MS/MS). Histamine-d4 (Toronto Research Chemicals) was used as internal standard. Inter-assay imprecision (n = 20 days) was < 2.9 % at three different levels (60, 986, 3873 nmol/L, respectively) and limit of quantification was 3.0 nM. Histamine release was calculated as percentage of total histamine present in both supernatant and slice. Cysteinyl leukotriene (CLT)

concentration of released supernatant was measured by ELISA (Cayman Chemical) according to the manufacturer's protocol.

Measurement of nitric oxide production

Airway NO production was visualized and quantified using the cell permeable NO probe 4,5-diaminofluorescein (DAF-2 DA; Abcam) and fluorescent imaging. PCLS were incubated with DAF-2 DA (10 μ M) for 30 min at in a humid atmosphere under 5% CO₂/95% air at 37°C, slices without DAF-2 DA were taken as a negative control. PCLS were washed four times every 5 min. Afterwards, PCLS were treated with ABH (1 μ M) for 30 min or left untreated. DAF-2 DA loaded PCLS were challenged with OA (10 μ g/ml) for 5 min. Then, all PCLS were fixed with paraformaldehyde (2%) for a minimum of 1h at room temperature. Following fixation, PCLS were placed on a glass slide and coverslips were mounted with ProLong Gold antifade reagent (Invitrogen). Using a confocal microscope (TCS; SP8 Leica, Heidelberg, Germany) fluorescence images were taken (maximal excitation λ = 491 nm, maximal emission λ = 513 nm). ImageJ 1.52i was used to further process images (Schindelin et al., 2012). The airway area was selected as region of interest.

In vitro biochemical arginase assay

The arginase inhibitors were tested for their ability to inhibit rhArg 1 and 2 *in vitro* via a colorimetric measurement of urea production (Golebiowski et al., 2013; Van Zandt et al., 2013). All dilutions except stop mix were made in assay buffer containing 130.0 mM NaCl, 80.9 mM Na₂HPO₄·2H₂O, 18.8 mM Na₂H₂PO₄·H₂O (pH=7.4) and 1 mg/mL OA. The assays were performed in 96-wells plates with a final volume of 60 μ l per well for each reaction. rhArg1 (R&D Systems, Minneapolis, MN, USA) or rhArg2 (United States Biological, Swampscott, MA, USA) (0.67 μ g/ml) were pre-incubated with arginase inhibitor (0.0167, 0.167, 1.67, 16.7 or 167 μ M) for 30 min at 37°C. Wells without inhibitor and wells without enzyme were used

as positive and negative controls, respectively. Urea was used as a standard. The reactions were started by adding 10 μ l substrate buffer (assay buffer supplemented with 120.0 mM L-arginine, 89.9 mM glycine and 1.80 mM MnCl_2) and incubated for 1h at 37°C. After 1h, the reactions were quenched using freshly prepared stop mix (130 mM boric acid, 2.8 M sulfuric acid (95-97%), 1.4% (v/v) Brij-35, 9.98 mM in DMSO-dissolved o-phthaldialdehyde (OPA), and 1.30 mM in DMSO-dissolved primaquine bisphosphate (PD)). The colorimetric reactions were allowed to develop for 10 min at room temperature. Optical density was measured using a BioTek platereader (Synergy H1 Hybrid Reader, BioTek®) at a wavelength of 530 nm. Arginase activity in these samples was defined as the urea production in 1h and expressed as percentage of the positive control (enzyme only). The compounds' potencies to inhibit urea production by rhArg1 and rhArg2 enzymes were determined and the concentrations causing half maximal inhibition (IC_{50}) were calculated.

Data analysis

In vitro and *ex vivo* data are represented as mean \pm SEM, *in vivo* data as individual results and median. The statistical significance of differences between means and medians was calculated by Student's t-test, One or Two Way ANOVA with Newman-Keuls post hoc test, as appropriate. Differences were considered to be statistically significant when $p < 0.05$.

7. Results

Arginase inhibition is bronchoprotective in ex vivo and in vivo guinea pig models of asthma

We first determined the effect of arginase inhibition *in vivo* in a guinea pig model of acute allergic asthma. To assess the response to allergen, animals were challenged until obstruction with OA. The animals' lung function (P_{pl}) was measured continuously for 6 hours and the magnitude of the EAR was calculated as the area under the curve. As shown in figure 1A, OA challenge in saline pretreated animals resulted in an EAR ($p < 0.001$) compared to saline

challenged animals. Pretreatment with ABH almost completely prevented the development of the OA-induced EAR ($p < 0.01$).

As the *in vivo* model would not be suitable for an extensive screening of pharmacologically active compounds in the initial phases, an *ex vivo* model of allergen-induced airway narrowing using PCLS was used. In the PCLS model, intrapulmonary airway responses can be studied in an intact lung microenvironment (Martin et al., 1996; Ressmeyer et al., 2006). OA induced dose-dependent narrowing of the intrapulmonary airways in PCLS obtained from OA-sensitized guinea pigs with a maximal response (E_{max}) of $81.9 \pm 3.7\%$ closure and a sensitivity to OA (EC_{50}) of $0.90 \pm 0.36 \mu\text{g/ml}$. Treatment of lung slices with the arginase inhibitor ABH resulted in a significant inhibition of OA-induced airway narrowing compared to controls ($p < 0.001$) (Figure 1B). ABH significantly reduced the E_{max} to $48.9 \pm 2.8\%$ ($p < 0.001$) compared to control. Furthermore, this was associated with a reduced sensitivity (EC_{50}) of the allergen-induced airway closure after treatment with ABH ($0.90 \pm 0.36 \mu\text{g/ml}$ for control vs $4.57 \pm 2.8 \mu\text{g/ml}$ in the presence of ABH; $p < 0.01$). Whereas treatment with the NOS inhibitor L-NAME alone did not affect OA-induced airway narrowing, L-NAME reversed the protective effect of ABH ($p < 0.001$) indicating a role for NO. These results are consistent with our previous reports (Maarsingh et al., 2008; Sopi et al., 2012).

To study in how far the protective effects of ABH in the PCLS may be caused by reducing mast cell degranulation, histamine levels in supernatants of lung slices were measured by LC-MS/MS. As depicted in figure 1C, histamine secretion dose-dependently increased after OA challenge ($p < 0.05$), which was significant at 10 and 100 $\mu\text{g/ml}$ OA ($p < 0.01$ and $p < 0.001$, respectively) compared to spontaneous histamine release. At 100 $\mu\text{g/ml}$ OA, 1 μM ABH tended to reduce the release of histamine, but this was not statistically significant. Also, higher concentrations of the inhibitor (10 and 100 μM) did not reduce OA-induced histamine release (Figure 1D). In addition to histamine, mast cells produce CLTs that may contribute to the

observed allergen-induced airway closure. In contrast to histamine, CLTs are not preformed and stored in mast cell granules, but synthesized in response to IgE receptor cross-linking (Montuschi, 2010). Levels of CLTs (LTC₄/D₄/E₄) in the supernatant of OA-challenged PCLS were not affected by arginase inhibition (Figure 1E).

Locally produced NO derived from cNOS is involved in the regulation of airway tone (Meurs et al., 2000). To verify that NO production in challenged airways was indeed increased after arginase inhibition, airway-specific NO production was visualized and quantified using the NO probe DAF-2 DA. DAF-2 DA is cell-permeable and directly reports the intracellular NO levels (Bradding et al., 2006). Allergen challenge reduced fluorescence intensity in the airways of guinea pig PCLS (Figure 1H). Furthermore, as illustrated in Figure 1F and 1G, and quantified in Figure 1H, ABH pre-treated PCLS show a higher fluorescence intensity in the airways after allergen challenge compared to untreated allergen-challenged slices ($p < 0.05$), indicating an increased NO production in the wall of the airways after arginase inhibition. These results show that arginase inhibition increases NO production in the airway wall, presumably by increasing the bioavailability of L-arginine for NOS, and thus protecting from allergen-induced airway narrowing. This confirms the suitability of the PCLS system for its use in the screening of novel arginase inhibitors.

Effects of current potent arginase inhibitors

After validating the protective effect of arginase inhibition in allergen-induced airway narrowing, we set out to test arginase inhibitors in our model. First the *in vitro* and *ex vivo* efficacy of the well-known reference compound ABH was compared with the efficacy of a recently published novel arginase inhibitor Mars' compound (Golebiowski et al., 2013). Using a colorimetric *in vitro* biochemical assay, the compounds' potencies to inhibit urea production by rhArg1 and rhArg2 enzymes were determined and the IC₅₀-values were calculated. ABH and

Mars' compound are both able to completely inhibit urea production by arginase 1 (Figure 2A) and arginase 2 (Figure 2B) in a dose-dependent manner. Moreover, Mars compound had slightly lower IC₅₀-values for both arginase isoenzymes than ABH.

Next, PCLS were pretreated with the arginase inhibitors and allergen dose-response curves were established. As expected, the OA-induced narrowing of intrapulmonary airways could be inhibited in a concentration-dependent manner by pretreatment with ABH (Figure 2C). This was associated with a decreased maximal effect (E_{max}) to OA by 1 μM and 10 μM ABH (p<0.001) and a decreased sensitivity (EC₅₀) to OA by all three concentrations (p<0.05) compared to control. Pretreatment of PCLS with Mars' compound (1 μM) resulted in a significant inhibition of airway narrowing (p<0.001) and increased EC₅₀ (p<0.05) compared to control, without affecting the E_{max} (Figure 2D).

In vitro and ex vivo potency of novel arginase inhibitors

In our search for novel arginase inhibitors >200 unique compounds were designed and synthesized. The novel arginase inhibitors were screened for their functional efficacy, by determining their potency to inhibit arginase *in vitro*, followed by the assessment of the *ex vivo* potency to inhibit allergen-induced airway narrowing in guinea pig PCLS for the most promising compounds. Figure 3 shows an example of the inhibitory effects on rhArg1 of ABH and several novel arginase inhibitors, expressed as the ability to inhibit urea production by arginase 1 and the associated IC₅₀-values. All compounds, with the exception of SHK099, were effective in the lower to medium micromolar range. Based on the *in vitro* efficacy, selected compounds were tested *ex vivo* in guinea pig PCLS in three different concentrations. This selection contained racemic compounds as well as enantiomers.

The racemic compound SHK081-5 was separated into its enantiomers SHK277 and SHK278. As shown in Figure 4A and B, the racemic compound and its enantiomers had similar IC₅₀-

values for the *in vitro* inhibition of both arginase 1 and 2. The racemic SHK081-5 protected against maximal OA-induced airway narrowing in PCLS in a concentration-dependent manner (Figure 4C), which was significant after pretreatment with 1 μ M, 10 μ M and 100 μ M SHK081-5 ($p < 0.001$) compared to control. The enantiomers SHK277 and SHK278 also reduced allergen-induced airway narrowing (Figure 4D and E). Interestingly, these compounds were already able to induce significant protection on OA-induced airway narrowing at 0.01 μ M, and significantly decreased E_{max} to OA at 0.1 μ M ($p < 0.05$), 1 μ M and 10 μ M SHK277 ($p < 0.01$), and 0.1 μ M and 1 μ M SHK278 ($p < 0.001$) compared to control. Furthermore, pretreatment with 0.1 μ M, 1 μ M and 10 μ M SHK278 significantly increased EC_{50} -values for OA compared to control.

The *in vitro* and *ex vivo* efficacies of the two enantiomers of SHK186-6, SHK242 and SHK243, are shown in Figure 5. SHK242 completely inhibited rhArg1 and rhArg2 with IC_{50} -values of 3.4 and 2.9 μ M, respectively. By contrast, the other enantiomer, SHK243, hardly inhibited rhArg1 and only partially inhibited rhArg2 and showed IC_{50} -values of 36.4 μ M for arginase 1 and 5.0 μ M for arginase 2 (Figure 5A and B). In comparison, the racemic compound SHK186-6 showed an IC_{50} -value of 22.5 μ M for rhArg1 (Figure 3). *Ex vivo* both enantiomers effectively suppressed OA-induced maximal airway narrowing (Figure 5C and 5D); furthermore 10 μ M SHK242 ($p < 0.05$) and 0.1 μ M SHK243 ($p < 0.001$) were able to significantly increase EC_{50} to OA compared to control.

In vivo potency of novel arginase inhibitors

We evaluated the *in vivo* efficacy of SHK242 and SHK277 in a guinea pig model of acute allergic asthma, comparing them to ABH. Using this model, the effect of the novel arginase inhibitors on allergen-induced changes in lung function and inflammation was studied. In order to measure the effect of the arginase inhibitors on AHR to histamine after allergen challenge, PC_{100} -values were assessed and AHR after the EAR and LAR was calculated. As shown in

Figure 6A, OA challenge resulted in the development of AHR after the EAR compared to saline challenge ($p < 0.001$). Pretreatment with ABH, SHK242 or SHK277 was not able to prevent allergen-induced AHR. No AHR was observed after the LAR in all groups (Figure 6B).

Also, the magnitudes of the EAR and LAR were quantified. Unfortunately, some animals got disconnected from the external canula during measurement of the EAR and LAR, and therefore not all animals could be included in the analyses. As shown in Figure 6C, OA challenge resulted in a significant EAR compared to saline challenge ($p < 0.001$). Pretreatment of the animals with the arginase inhibitors ABH, SHK242 or SHK277 (25 mM, nebulizer concentrations) protected against the EAR compared to saline-treated animals ($p < 0.05$ for ABH and SHK277, and $p < 0.001$ for SHK242). Similarly, OA challenge induced a significant LAR in the saline-treated animals ($p < 0.001$), whereas treatment with ABH, SHK242 or SHK277 protected from this increase ($p < 0.001$; Figure 6D).

One hour after measuring the LAR, the animals underwent bronchial alveolar lavage to study allergen-induced inflammatory cell infiltration in the lungs. OA challenge increased the numbers of total inflammatory cells ($p < 0.01$) and eosinophils ($p < 0.05$) compared to saline challenged animals. Treatment with the arginase inhibitors did not affect total inflammatory cell number or eosinophils (Supplemental Figure 1).

8. Discussion

The current study set out to evaluate the pharmacological efficacy of a novel class of arginase inhibitors for the potential treatment of allergic asthma. We synthesized and validated a group of novel compounds for their *in vitro* efficacies to inhibit arginases and their protective effects in *ex vivo* and *in vivo* models of allergic asthma. We showed that our arginase inhibitors are able to inhibit recombinant human arginases *in vitro*. The separated enantiomers are able to protect against allergen-induced airway narrowing *ex vivo* in guinea pig lung slices and, similar

to ABH, are able to inhibit the development of the EAR and LAR in an *in vivo* guinea pig model of acute allergic asthma. This shows that we have identified promising novel arginase inhibitors for the potential treatment of allergic asthma, which was the primary aim of our studies.

By comparing the reference compound ABH to the patented Mars' compound, we showed that a high *in vitro* efficacy of an arginase inhibitor does not necessary translate into a high *ex vivo* efficacy as – in contrast to ABH – Mars' compound did not reduce the E_{max} (Figure 2). These differences may be explained by differences in the bio-availability of these relatively hydrophilic compounds which may inhibit effective transmembrane uptake. Our study thus points out an important difference between the *in vitro* target binding efficacy of an arginase inhibitor and the *ex vivo* and *in vivo* efficacy where tissue penetration and cell uptake considerably affect compound efficacy.

A secondary aim of our studies was to investigate the mechanism of action of arginase inhibition in allergen-induced responses in more detail. Allergen-induced airway narrowing is mast cell dependent. By releasing their pro-contractile mediators, mast cells form an important link between allergen encounter and airway smooth muscle contraction (Bradding et al., 2006). It was shown that NO modulates allergen-induced histamine release in rat peritoneal mast cells (Brooks et al., 1999; Maarsingh et al., 2008). As arginase inhibition affects allergen-induced airway responses in guinea pig models (Maarsingh et al., 2008), we hypothesized that this protective effect of arginase inhibition on allergic airway narrowing by is caused by increasing NO synthesis, thereby inhibiting mast cell degranulation. However, our present results indicate that this is unlikely as in PCLS no effect of arginase inhibition on allergen-induced histamine or CLT release was observed, suggesting that mast cell independent processes are involved. We did show that arginase inhibition protects against allergen-induced airway narrowing via a mast cell-independent, NO-dependent mechanism. In the present study we observed that OA challenge leads to a reduction of NO in the airway wall compared to basal NO levels. It is

known that locally produced NO derived from cNOS is involved in the regulation of airway tone (Meurs et al., 2002). In both animal models (de Boer et al., 1996; De Boer et al., 1999) and asthmatic patients (Ricciardolo et al., 2001; Ricciardolo et al., 2008; Silkoff et al., 2000) insufficient production of NO by cNOS is involved in the pathophysiology of allergic asthma. In guinea pigs, we demonstrated that this deficiency is caused by allergen-induced increased arginase activity that causes substrate limitation to the enzyme (Meurs et al., 2002). Inhibition of arginase will lead to increase of substrate for the formation of NO by cNOS. The visualized increased NO levels in the airway wall in our study support pharmacological data obtained in this and previous studies. Previously, it was shown that nor-NOHA and ABH could reverse methacholine-induced airway narrowing and allergen-induced AHR in perfused guinea pig trachea preparations (Maarsingh et al., 2009). Reversal of this bronchodilating effect of arginase inhibition was achieved by co-incubation with L-NAME, thus showing arginase inhibition increases NO production. A similar mechanism was shown to underlie arginase inhibitor-induced relaxation of inhibitory nonadrenergic non-cholinergic nerves in airway smooth muscle (Maarsingh et al., 2006).

Our *ex vivo* and *in vivo* results were obtained using guinea pig models. Guinea pigs are often used to study allergic asthma as they are thought to share both pharmacological and physiological features with humans (Muccitelli et al., 1987; Popa et al., 1973; Ricciardolo et al., 2008). Furthermore, we and others have shown that guinea pig PCLS can be used as a model for both allergic asthma and COPD, as they share similar features with PCLS obtained from patients (Maarsingh et al., 2019; Ressmeyer et al., 2006). In this study we have shown that the reference arginase inhibitor ABH and novel inhibitors SHK242 and 277 protect against the development of the EAR and LAR in a guinea pig model of acute allergic asthma, without affecting AHR to inhaled histamine after the EAR. However, we did not observe an inhibitory effect of arginase inhibition on airway inflammatory cell infiltration. The *in vivo* results differ

slightly from our previous study where we showed that next to decreasing the magnitude of the EAR and LAR, arginase inhibition with ABH can also inhibit allergen-induced AHR after the EAR and LAR in guinea pigs (Maarsingh et al., 2008). Important to note, however, are the differences in the experimental set-up between the two studies. In the previous study, the animals were treated with ABH twice: once 30 minutes before and once 8 hours after allergen-challenge, whereas in the current study the animals were challenged and treated only once 30 minutes before allergen challenge. In addition, in the current study we used a different strain of guinea pigs than in the previous study, due to the fact that the original strain was discontinued. Moreover, in the previous study each animal served as its own control as animals were all treated with vehicle in week 1 and with ABH or vehicle in week 2. Given differences in the protocols, we included ABH again so we could directly compare the efficacy of the novel arginase inhibitors to the highly effective reference compound ABH. In this regard, we have shown that SHK242 and SHK 277 are at least equally potent and efficacious to protect against allergen-induced airway responses *ex vivo* and *in vivo* as ABH. Despite all efforts, there are currently no arginase inhibitors available for clinical use and the inhibitors often used for research, such as ABH and nor-NOHA, show poor pharmacokinetic profiles (Pudlo et al., 2017). Therefore, research into the development and efficacy of novel arginase inhibitors is still necessary. The current study introduces and evaluates two novel arginase inhibitors SHK242 and SHK277 that are at least equally potent and efficacious in protecting against allergen-induced airway responses *ex vivo* and are at least equally effective as ABH *in vivo*. Compared to ABH, the chances of side-effects are expected to be even lower for our novel compounds, since they have more elaborate structures that do not only bind to the active side of arginase, but also to other binding pockets. Furthermore, another advantage of the novel compounds is that the synthesis of the SHK-compound series is much easier and less time-consuming and thus less expensive compared to ABH (Morris, 2009).

Despite efforts in expanding the treatment options for asthmatic patients, there is still a group of patients, especially those with severe asthma, that do not respond adequately to the current therapeutics (Reddel et al., 2019). Chronic airway inflammation in patients with severe asthma results in a reduction of bronchoprotective cNOS-derived NO (Ricciardolo et al., 1997). Increased arginase activity reduces the substrate availability of cNOS and thus the production of cNOS-derived NO (Maarsingh et al., 2006; Maarsingh et al., 2009). Local and systemic changes in arginase and arginine levels are observed in asthmatic patients and can be linked to among others asthma severity and lung function (Maarsingh et al., 2009; Xu et al., 2017), making arginase an interesting potential target for the treatment of asthma. Numerous studies in both cell and animal models indicate the potential usefulness of arginase inhibitors in various diseases as reviewed in (Pudlo et al., 2017). Furthermore, small-scale clinical studies locally applying arginase inhibitors already showed promising results in patients with heart failure (Quitter et al., 2013), hypertension (Holowatz and Kenney, 2007), coronary heart disease (Kövamees et al., 2014), coronary heart disease and diabetes (Shemyakin et al., 2012), familial hypercholesterolaemia (Kovamees et al., 2016), and cardiopulmonary resuscitation (Jung et al., 2014). Initial concerns about toxic side effects, especially with regard to ammonia detoxification in the liver, were mitigated by the lack of toxic side effects in animal models of hypertension and atherosclerosis after both short-term and long-term systemic treatment with arginase inhibitors (Pernow and Jung, 2013). The absence of these side effects could most likely be explained by the far higher expression levels of arginase in the liver as compared to vessels, resulting in the observation that low circulating concentrations of arginase inhibitors are sufficient to improve vascular function, but not high enough to significantly suppress liver arginase activity (Pernow and Jung, 2013). Moreover, many diseases are currently treated by local drug administration, as it requires lower doses of drugs and reduces the chance of side-

effects. Lung diseases, such as allergic asthma, can be treated locally by inhalation of nebulized drugs.

To conclude, in the current study we have identified novel potent arginase inhibitors for the potential treatment of allergic asthma. Our inhibitors are able to inhibit arginases *in vitro* and protect against allergic airway responses *ex vivo* and *in vivo*. This protection appears to be primarily mediated by an increase of bronchodilating NO production in the airways, leading to a reduced airway response. Based on the recent observations that arginase is importantly involved in particularly severe asthma, our findings hold promise for the treatment of these patients.

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10. Authorship contributions

- o Participated in research design: MB, HMa, LK, IK, PE, AD, HMe and RG
- o Conducted experiments: MB, SE, IH, PB, and MF
- o Contributed new reagents or analytic tools: SK, IK and AD
- o Performed data analysis: MB
- o Wrote or contributed to the writing of the manuscript: MB, SK, HMe and RG

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12. Footnotes

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Conflict of interest

HMa and HMe are inventors on a patent on the application of arginase inhibitors for the use in asthma and allergic rhinitis (US12/515,866). The study was partly funded by Carmolex BV who licensed in this patent. A patent application (EPO 19180160.2) is pending for the arginase inhibitors described in this paper, on which SK and AD are inventors.

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13. Figure legends

Figure 1: Arginase inhibition affects allergen-induced airway narrowing in allergic guinea pigs. (A) Effects of inhaled saline or ABH on allergen (OA)-induced early asthmatic reaction (EAR). A group of saline-treated, saline-challenged guinea pigs serves as control. The magnitude of the EAR is presented as area under the P_{pl} time-response curve between 0 and 6 hours after allergen challenge. Data represent medians of 6-10 animals. Statistical difference of the medians was determined by One Way ANOVA with Newman-Keuls post hoc test. *** $p < 0.001$ compared to saline challenged-saline treated (Sal/Sal) animals, ## $p < 0.01$ compared to OA challenged-saline treated (OA/Sal) animals. (B) OA dose response curves in untreated and arginase inhibitor ABH (1 μM) and/or NOS inhibitor L-NAME (0.1 mM) pretreated PCLS of OA-sensitized guinea pigs. Statistical difference of the means was determined by Two Way ANOVA with Newman-Keuls post hoc test. Data represents mean \pm SEM of 5 animals. *** $p < 0.001$ compared to control. ### $p < 0.001$ compared to ABH. (C) OA (0.1; 1; 10 or 100 μM) -induced histamine release of untreated and ABH (1 μM) -pretreated PCLS of OA-sensitized guinea pigs. Histamine release was calculated as % of total histamine level in both slice and supernatant. Statistical difference of the means was determined by Two Way ANOVA with Newman-Keuls post hoc test. Data represents mean \pm SEM of 5 animals. ** $p < 0.01$ and *** $p < 0.001$ compared to spontaneous histamine release (dotted line); n.s., not significant. (D) OA (10 μM)-induced histamine release of untreated PCLS and PCLS pretreated with different concentrations of ABH (1; 10; 100 μM). Data represents mean \pm SEM of 6 animals. (E) OA (10 μM)-induced cysteinyl leukotriene (CLT) release of untreated and ABH pretreated PCLS of OA-sensitized guinea pigs. Dotted line indicates spontaneous release. Statistical difference of the means was determined by One Way ANOVA with Newman-Keuls post hoc test. Data represents mean \pm SEM of 5 animals. Visualisation of NO production in the airways in response to OA-challenge (10 μM) in (F) untreated and (G) ABH (1 μM) pretreated guinea pig PCLS

using the fluorescent probe DAF-2DA. Produced NO is discernable in green at the airways (A), blood vessel (V) and lung tissue. **(H)** Quantification of mean fluorescence intensity in the airways. Data represents mean \pm SEM in 7 animals. Statistical difference of the means was determined by Student's t-test. * $p < 0.05$ compared to OA. Abbreviations: OA, ovalbumin; Sal, saline; EAR, early asthmatic reaction; ABH, 2(S)-amino-6-boronoheptanoic acid; L-NAME, N(ω)-nitro-L-arginine methyl ester; CLTs, cysteinyl leukotrienes; V, vessel; A, airway; A.U., arbitrary unit. AUC_{pl}, area under the pleural pressure-time curve.

Figure 2: Effects of the reference compounds ABH and Mars' compound. *In vitro* efficacy of ABH and Mars' compound to inhibit recombinant human arginase 1 **(A)** and recombinant human arginase 2 **(B)**. Data represents mean \pm SEM of 3-5 independent experiments. *Ex vivo* efficacy of ABH (0.1, 1 and 10 μ M) **(C)** or ABH (1 μ M) and Mars' compound (1 μ M) **(D)** to inhibit allergen (OA)-induced airway narrowing. OA dose response curves in untreated and arginase inhibitor pretreated PCLS of OA-sensitized guinea pigs. Data is presented as mean \pm SEM of 5-18 animals. Statistical difference of the means was determined by One or Two way ANOVA with Newman-Keuls post-hoc test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control, # $p < 0.05$ compared to ABH treated. Abbreviations: ABH, 2(S)-amino-6-boronoheptanoic acid; IC₅₀, half maximal inhibitory concentration; E_{max}, maximal effect; EC₅₀, half maximal effective concentration.

Figure 3: *In vitro* efficacy of selected novel arginase inhibitors. *In vitro* efficacy of several novel compounds to inhibit recombinant human arginase 1. Data represents means \pm SEM of 3-6 independent experiments. Abbreviations: ABH, 2(S)-amino-6-boronoheptanoic acid; IC₅₀, half maximal inhibitory concentration.

Figure 4: *In vitro* and *ex vivo* efficacy of the novel racemic compound SHK081-5 and its enantiomers SHK277 and SHK278. *In vitro* efficacy of SHK081-5, SHK277 and SHK278 to inhibit recombinant human arginase 1 (A) and recombinant human arginase 2 (B). Data represents means \pm SEM of 3-6 independent experiments. *Ex vivo* efficacy of SHK081-5 (1, 10 and 100 μ M) (C), SHK277 (0.01, 0.1, 1 and 10 μ M) (D) and SHK278 (0.01, 0.1, 1 and 10 μ M) (E) to inhibit ovalbumin (OA)-induced airway narrowing in PCLS of OA-sensitized guinea pigs. Data is presented as mean \pm SEM of 5-7 animals. Statistical difference of the means was determined by One or Two way ANOVA with Newman-Keuls post-hoc test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control. Abbreviations: ABH, 2(S)-amino-6-boronoheptanoic acid; IC₅₀, half maximal inhibitory concentration; E_{max}, maximal effect; EC₅₀, half maximal effective concentration; OA, ovalbumin.

Figure 5: *In vitro* and *ex vivo* efficacy of the novel enantiomers SHK242 and SHK243. *In vitro* efficacy of SHK242 and SHK243 to inhibit recombinant human arginase 1 (A) and recombinant human arginase 2 (B). Data represents mean \pm SEM of 4-6 independent experiments. *Ex vivo* efficacy of SHK242 (0.01, 0.1, 1 and 10 μ M) (C) and SHK243 (0.01, 0.1, 1 and 10 μ M) (D) to inhibit ovalbumin (OA)-induced airway narrowing in PCLS of OA-sensitized guinea pigs. Data is presented as mean \pm SEM of 5-6 animals. Statistical difference of the means was determined by One or Two way ANOVA with Newman-Keuls post-hoc test. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to control. Abbreviations: ABH, 2(S)-amino-6-boronoheptanoic acid; IC₅₀, half maximal inhibitory concentration; E_{max}, maximal effect; EC₅₀, half maximal effective concentration; OA, ovalbumin.

Figure 6: Effect of ABH and novel arginase inhibitors on allergen-induced AHR and allergic reactions. Effects of inhaled saline, ABH, SHK242 or SHK277 (25 mM all, nebulizer concentrations, 3 minutes) on allergen (OA)-induced AHR at 6h (A) or 24h (B) after OA challenge. Data are expressed as the ratio of the baseline histamine PC₁₀₀-value pre-challenge

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to the histamine PC₁₀₀-value at 6h (EAR) and 24 h (LAR) post-challenge and represented as medians of n=8-13 animals. Also the effects on the magnitude of EAR (**C**) and LAR (**D**) was measured. Data is presented as medians of n=5-12. Statistical difference was determined by One Way ANOVA on log transformed data with Newman-Keuls post hoc test. *p<0.05, ***p<0.001 compared to saline challenged-saline treated (Sal/Sal) animals. ##p<0.01 and ###p<0.001 compared to OA-challenged saline-treated (OA/Sal) animals. OA, ovalbumin; Sal, saline; AHR, airway hyperresponsiveness; EAR, early asthmatic reaction; LAR, late asthmatic reaction; ABH, 2(S)-amino-6-boronoheptanoic acid; AUC, area under the pleural pressure-time curve.

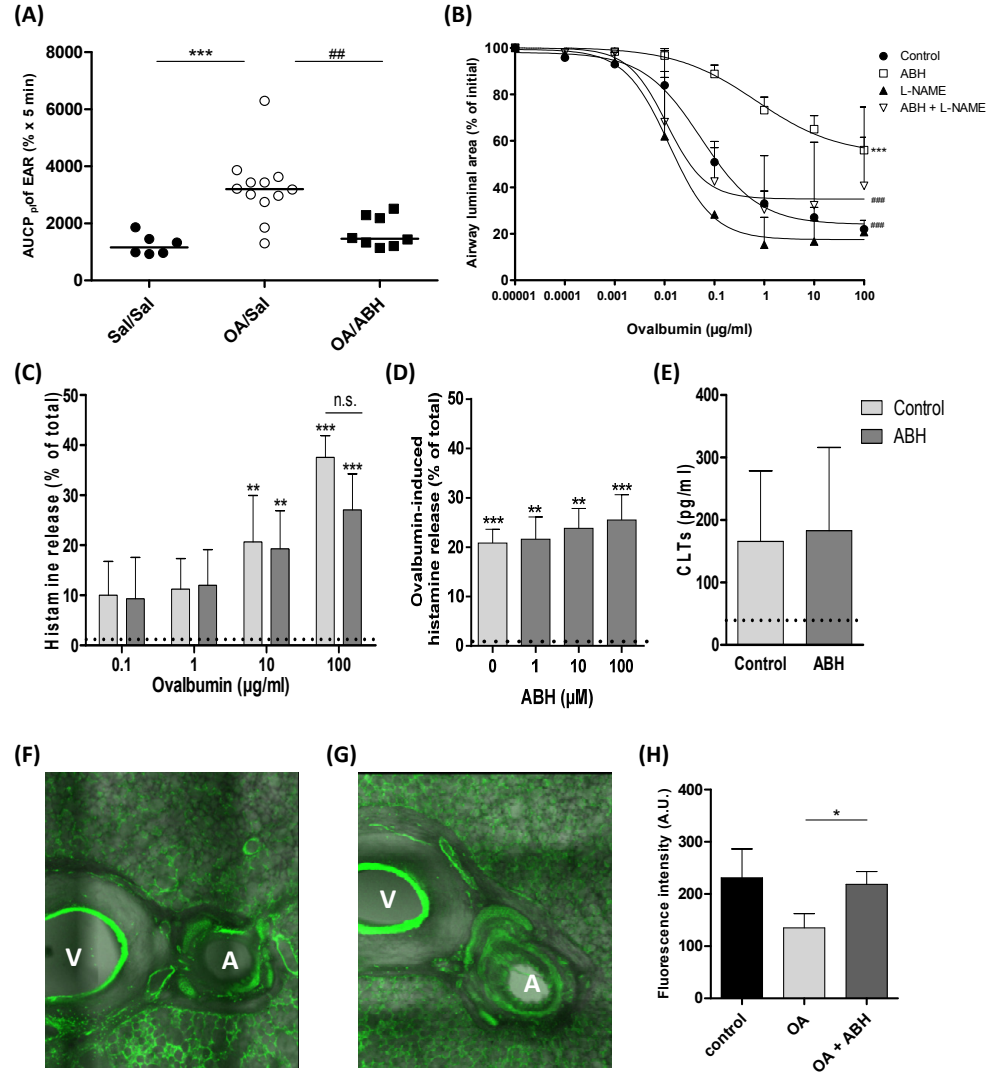


Figure 2

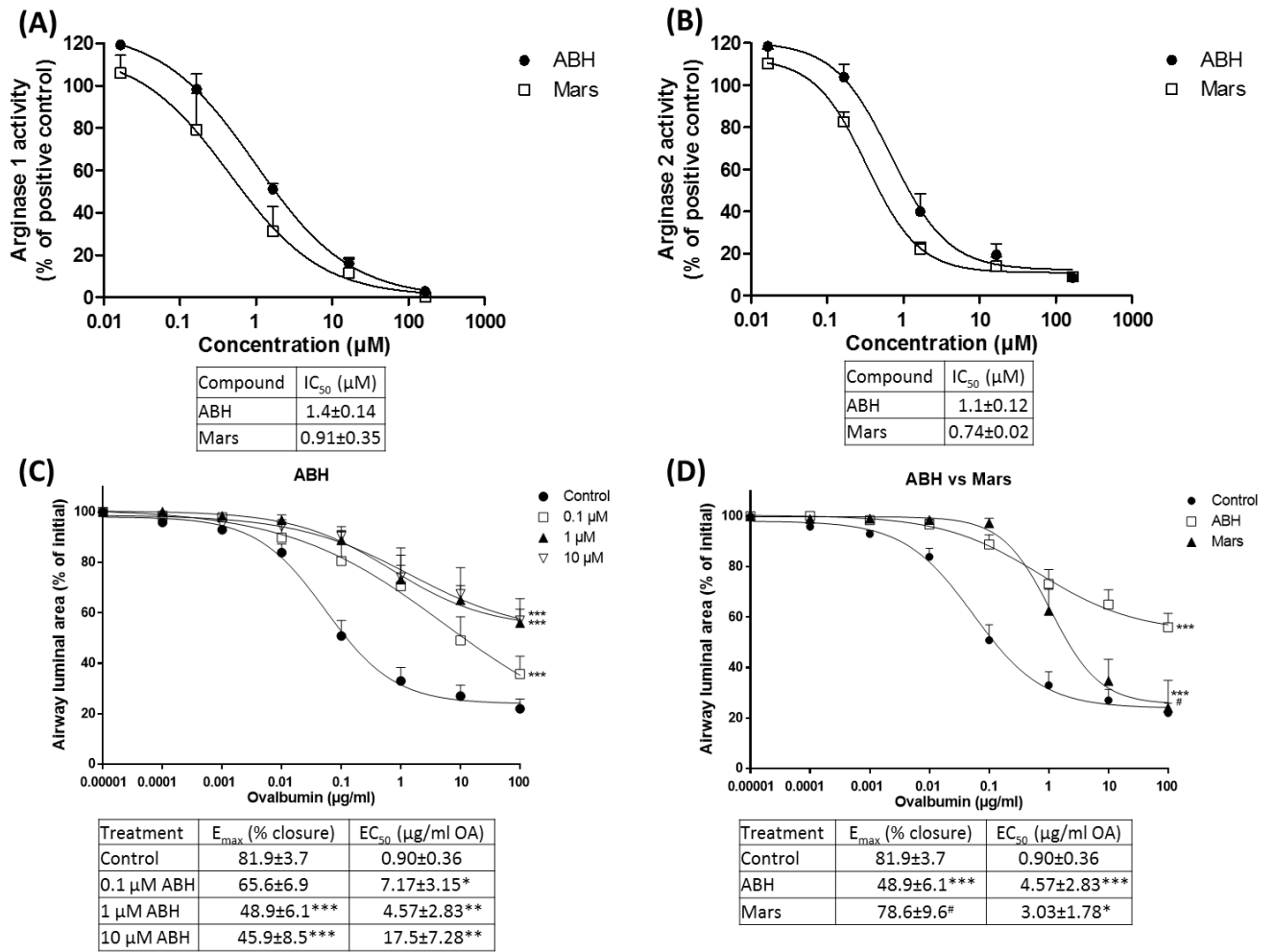


Figure 3

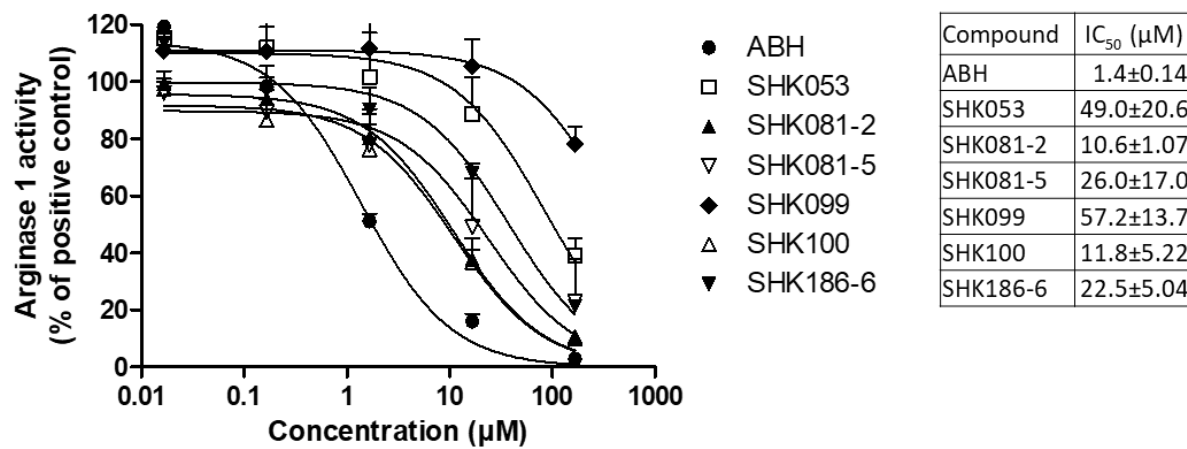


Figure 4

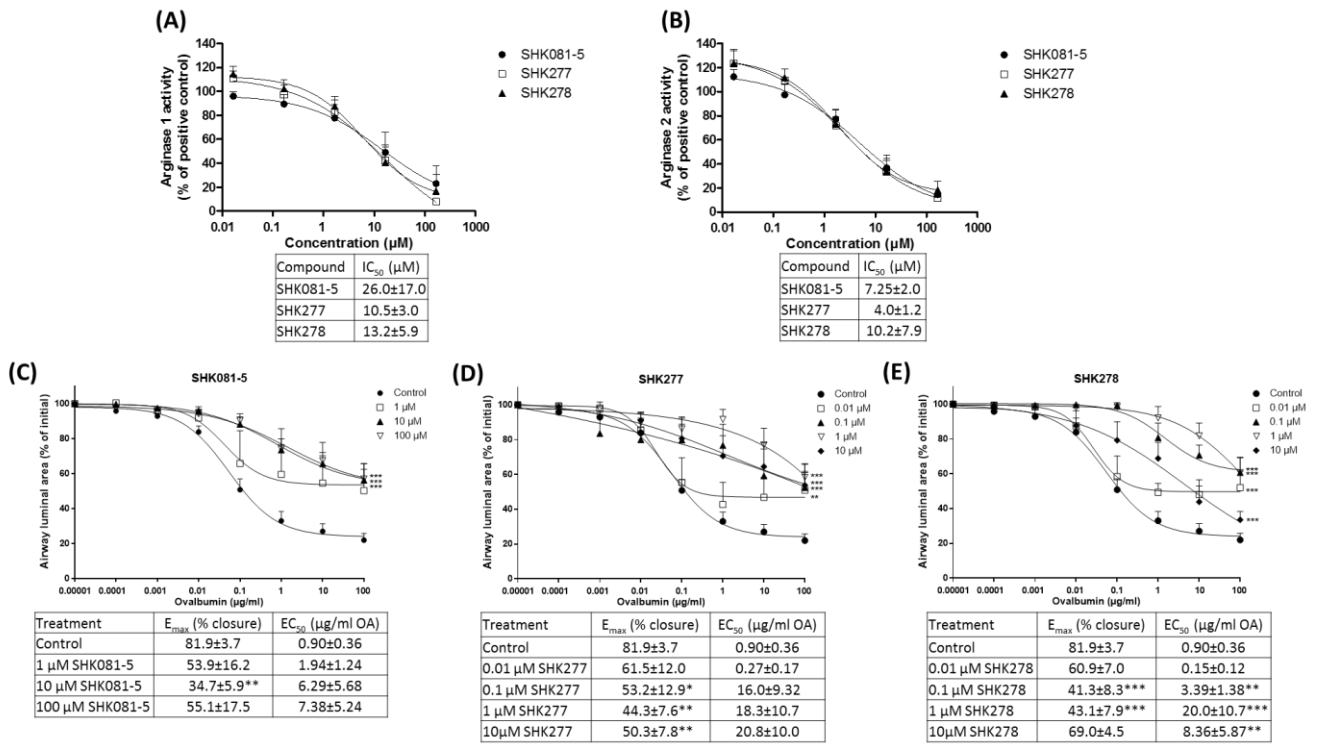


Figure 5

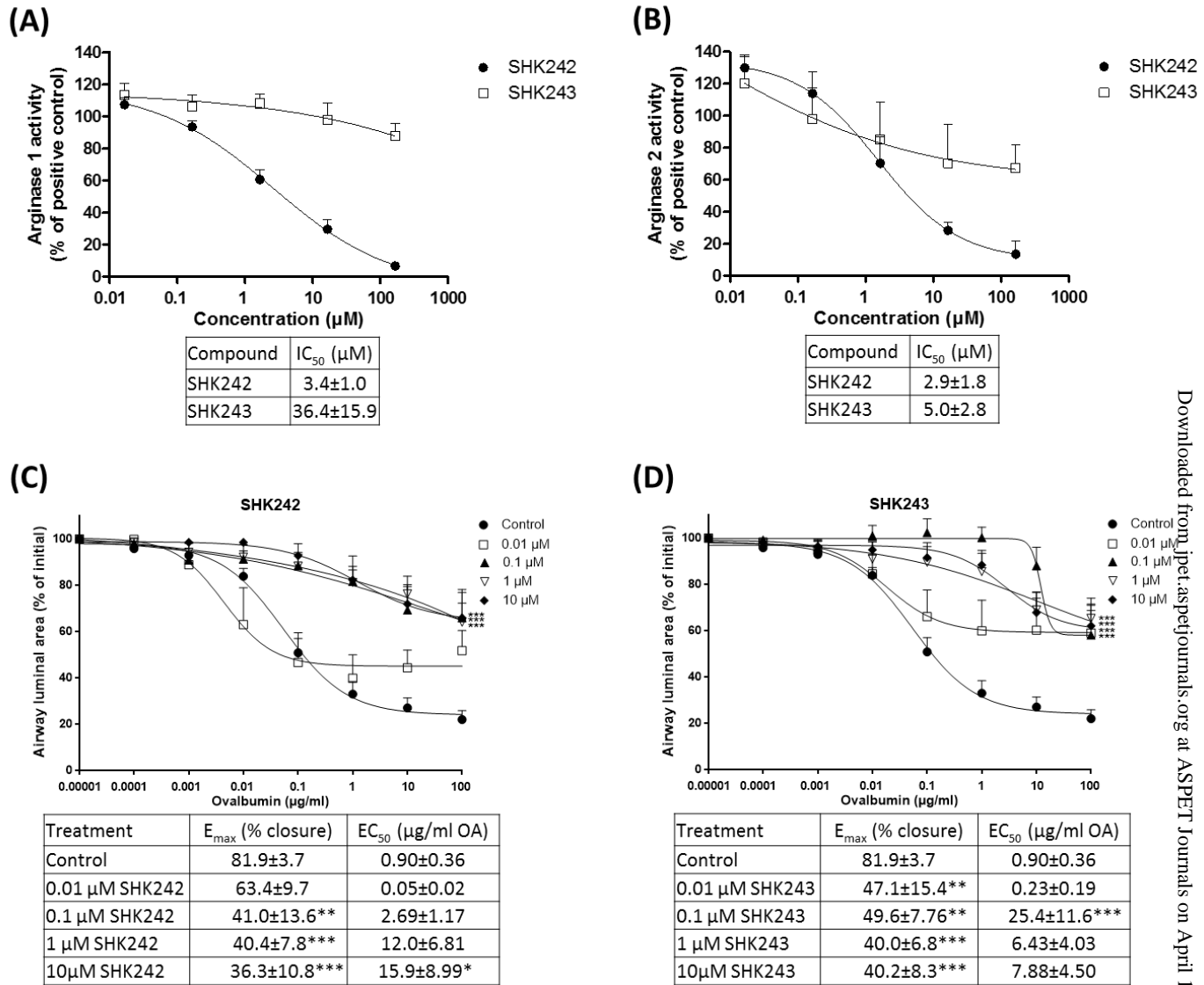


Figure 6

