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# Pharmacological Screening Identifies SHK242 and SHK277 as Novel Arginase Inhibitors with Efficacy against Allergen-Induced Airway Narrowing In Vitro and In Vivo[S]

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## ABSTRACT

Arginase is a potential target for asthma treatment. However, there are currently 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-boronohexanoic 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 hyper-responsiveness (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 $IC_{50}$ 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 with 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.

## 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.

## Introduction

Allergic asthma is a common chronic airway disease that affects millions of people worldwide. It is characterized by recurrent airway obstruction, airway hyper-responsiveness (AHR), airway inflammation, and airway remodeling. In patients with allergic asthma, exposure to inhaled allergens results in an early asthmatic reaction (EAR) driven by allergen-specific IgE, which binds to surface receptors on basophils and mast cells (Murray et al., 1985; Liu et al., 1991). Mast cell activation leads to release of proinflammatory

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H.Ma. and H.Me 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 S.H.K. and A.D. are inventors.

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**ABBREVIATIONS:** ABH, 2(S)-amino-6-boronohexanoic acid; AHR, airway hyper-responsiveness; CLT, cysteinyl leukotriene; cNOS, constitutive nitric oxide synthase; DAF-2 DA, 4,5-diaminofluorescein; EAR, early asthmatic reaction; $E_{max}$, maximal effect; IL, interleukin; LAR, late asthmatic reaction; L-NAME, N(ω)-nitro-L-arginine methyl ester; NO, nitric oxide; nor-NOHA, $N^{\omega}$-hydroxy-nor-L-arginine; NOS, nitric oxide synthase; OA, ovalbumin; $PC_{100}$, provocation concentration causing 100% increase of pleural pressure; PCLS, precision-cut lung slice; $P_{pl}$, pleural pressure; rhArg, recombinant human arginase.



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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 patients with asthma consists of a combination of inhaled anti-inflammatory corticosteroids and bronchodilating long-acting $\beta$2-adrenoceptor agonists (Barnes, 2011). More recently, the anticholinergic tiotropium, anti-IgE, anti–interleukin (IL)-5/5 receptor, and anti–IL-4 receptor have been added to the Global Initiative for Asthma 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 of 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 (Que et al., 1998; Maarsingh et al., 2009b). Their expression can be induced by proinflammatory agents such as T helper 2 (Th2)-cytokines and cigarette smoke (Maarsingh et al., 2009b). In the airways, there is competition between arginase and NOS for the common substrate L-arginine (Meurs et al., 2002; Maarsingh et al., 2006, 2009b). Even though the affinity of NOS for L-arginine is approximately 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 hyper-responsiveness (Maarsingh et al., 2008; North et al., 2009; Bratt et al., 2010).

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 allergen-challenged guinea pigs, we showed that arginase inhibition was able to reduce methacholine-induced contractions ex vivo (Meurs et al., 2002; Maarsingh et al., 2009a). 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 patients with asthma (Zimmermann et al., 2003; North et al., 2009). 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 $\beta_2$-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 ABH and $N^{\omega}$-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 with ABH. For assessing functional efficacy, their effect on allergen-induced airway narrowing in precision-cut lung slices (PCLSs) 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.

## Methods

**Arginase Inhibitors.** In total, >200 novel arginase inhibitors were developed using structure-based design (Anchor Query) and multicomponent reactions as described in Domling et al. (2012) and 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 analog 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 g 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 subcutaneous 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: June 4, 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 12-hour light/dark cycle and were provided ad libitum access to food and water and cage enrichment.

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**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 minutes, followed by a control provocation with saline lasting 3 minutes, 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 μg/ml) in saline were performed to assess the airway reactivity to histamine. Histamine provocations lasted maximally 3 minutes and were separated by 8-minute 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 toward histamine. Within 15 minutes 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 24 hours before OA challenge. Half an hour before OA challenge, the animals were treated via inhalation with the arginase inhibitors ABH, SHK242, or SHK277 (25 mM nebulizer concentration, 3 minutes) or saline as a control. Animals were challenged with OA (0.05%–0.1%) until obstruction. Six hours (after the EAR) and 24 hours (after the LAR) after challenge, AHR to histamine was determined (Meurs et al., 2006). Twenty-five hours after allergen challenge, a bronchial alveolar lavage was performed. Allergen-induced AHR was calculated as a ratio of histamine responsiveness before and after OA challenge. The magnitudes of the EAR and LAR were determined by measurement of the area under the $P_{pl}$-time curve between 0–6 and 6–24 hours, respectively.

**Bronchial Alveolar Lavage.** Animals were anesthetized 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, 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 minutes at 4°C. The pellets were combined and resuspended to a final volume of 1.0 ml in PBS, and total cell numbers were counted manually. Cytospin preparations were stained with May-Grünwald and Giemsa stain for cytologic 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.** PCLSs were prepared, as described previously (Ressmeyer et al., 2006; Maarsingh et al., 2019), 4–10 weeks after sensitization. Medium containing $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), KCl (5.4 mM), NaCl (116.4 mM), $NaH_2PO_4$ (1.2 mM), glucose (16.7 mM), $NaHCO_3$ (26.1 mM), Hepes (25.2 mM), and penicillin/streptomycin (5000 U/ml) (pH = 7.2) was prepared. Animals were euthanized by an intracutaneous (i.c.) injection of an overdose of pentobarbital (Euthasol 20%; Produlab Pharma, Raamsdonksveer, The Netherlands) and exsanguinated. 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), amino acids mixture (1:50), and vitamins mixture (1:100) was injected into the lungs. Ice was placed on the lungs for at least 30 minutes to solidify the agarose, after which the lungs were removed and placed on ice. The lobes were separated to prepare tissue cores with a diameter of 15 mm. Using a tissue slicer (CompresstomeTM VF-300 microtome; Precisionary Instruments, San Jose, CA), 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 PCLSs were incubated in a humid atmosphere under 5% $CO_2$/95% air at 37°C and washed every 30 minutes three 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.** PCLSs were used for OA-induced airway narrowing studies. PCLS were placed in wells containing 1 ml of supplemented medium at 37°C. A Teflon ring was used to fix the slices in position. PCLSs 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 (one frame per 2 seconds) of the PCLSs were captured. Using image acquisition software (NIS-elements; Nikon), the airway luminal area was quantified and expressed as the 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 Determination.** Untreated and ABH (1, 10, or 100 μM)-pretreated PCLSs were challenged with OA (0.1, 1, 10, 100 μg/ml) for 5 minutes to determine the effect of arginase inhibition on allergen-induced histamine release. Untreated, unchallenged PCLSs 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 seconds; 60 pulses; Vibra Cell; Sonics, Newton), and centrifuged for 30 minutes 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. Histamine-d4 (Toronto Research Chemicals) was used as internal standard. Interassay 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

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probe 4,5-diaminofluorescein (DAF-2 DA; Abcam) and fluorescent imaging. PCLSs were incubated with DAF-2 DA (10 μM) for 30 minutes in a humid atmosphere under 5% $CO_2$/95% air at 37°C, and slices without DAF-2 DA were taken as a negative control. PCLSs were washed four times every 5 minutes. Afterward, PCLSs were treated with ABH (1 μM) for 30 minutes or left untreated. DAF-2 DA–loaded PCLSs were challenged with OA (10 μg/ml) for 5 minutes. Then, all PCLSs were fixed with paraformaldehyde (2%) for a minimum of 1 hour at room temperature. Following fixation, PCLSs 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 $\lambda$ = 491 nm, maximal emission $\lambda$ = 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 rhArg1 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 $Na_2HPO_4{\cdot}2H_2O$, and 18.8 mM $Na_2H_2PO_4{\cdot}H_2O$ (pH = 7.4) and 1 mg/ml OA. The assays were performed in 96-well plates with a final volume of 60 μl per well for each reaction. rhArg1 (R&D Systems, Minneapolis, MN) or rhArg2 (United States Biologic, Swampscott, MA) (0.67 μg/ml) were preincubated with arginase inhibitor (0.0167, 0.167, 1.67, 16.7, or 167 μM) for 30 minutes 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 μl of substrate buffer (assay buffer supplemented with 120.0 mM L-arginine, 89.9 mM glycine, and 1.80 mM $MnCl_2$) and incubated for 1 hour at 37°C. After 1 hour, 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, and 1.30 mM in DMSO-dissolved primaquine bisphosphate]. The colorimetric reactions were allowed to develop for 10 minutes at room temperature. Optical density was measured using a BioTek plate reader (Synergy H1 Hybrid Reader; BioTek) at a wavelength of 530 nm. Arginase activity in these samples was defined as the urea production in 1 hour 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 means ± S.E.M., and in vivo data are represented as individual results and medians. The statistical significance of differences between data was calculated by Student's *t* test or one- or two-way ANOVA with Newman-Keuls post hoc test, as appropriate. Differences were considered to be statistically significant when $P < 0.05$.

## 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 Fig. 1A, OA challenge in saline-pretreated animals resulted in an EAR ($P < 0.001$) compared with 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 PCLSs obtained from OA-sensitized guinea pigs, with a maximal response ($E_{max}$) of 81.9% ± 3.7% closure and a sensitivity to OA ($EC_{50}$) of 0.90 ± 0.36 μg/ml. Treatment of lung slices with the arginase inhibitor ABH resulted in a significant inhibition of OA-induced airway narrowing compared with controls ($P < 0.001$) (Fig. 1B). ABH significantly reduced the $E_{max}$ to 48.9% ± 2.8% ($P < 0.001$) compared with control. Furthermore, this was associated with a reduced sensitivity ($EC_{50}$) of the allergen-induced airway closure after treatment with ABH (0.90 ± 0.36 μg/ml for control vs. 4.57 ± 2.8 μg/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 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 liquid chromatography in combination with isotope dilution tandem mass spectrometry. As depicted in Fig. 1C, histamine secretion dose-dependently increased after OA challenge ($P < 0.05$), which was significant at 10 and 100 μg/ml OA ($P < 0.01$ and $P < 0.001$, respectively) compared with spontaneous histamine release. At 100 μg/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 (Fig. 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 (Leukotriene (LT)C4/D4/E4) in the supernatant of OA-challenged PCLSs were not affected by arginase inhibition (Fig. 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 PCLSs (Fig. 1H). Furthermore, as illustrated in Fig. 1, F and G and quantified in Fig. 1H, ABH-pretreated PCLSs show a higher fluorescence intensity in the airways after allergen challenge compared with 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

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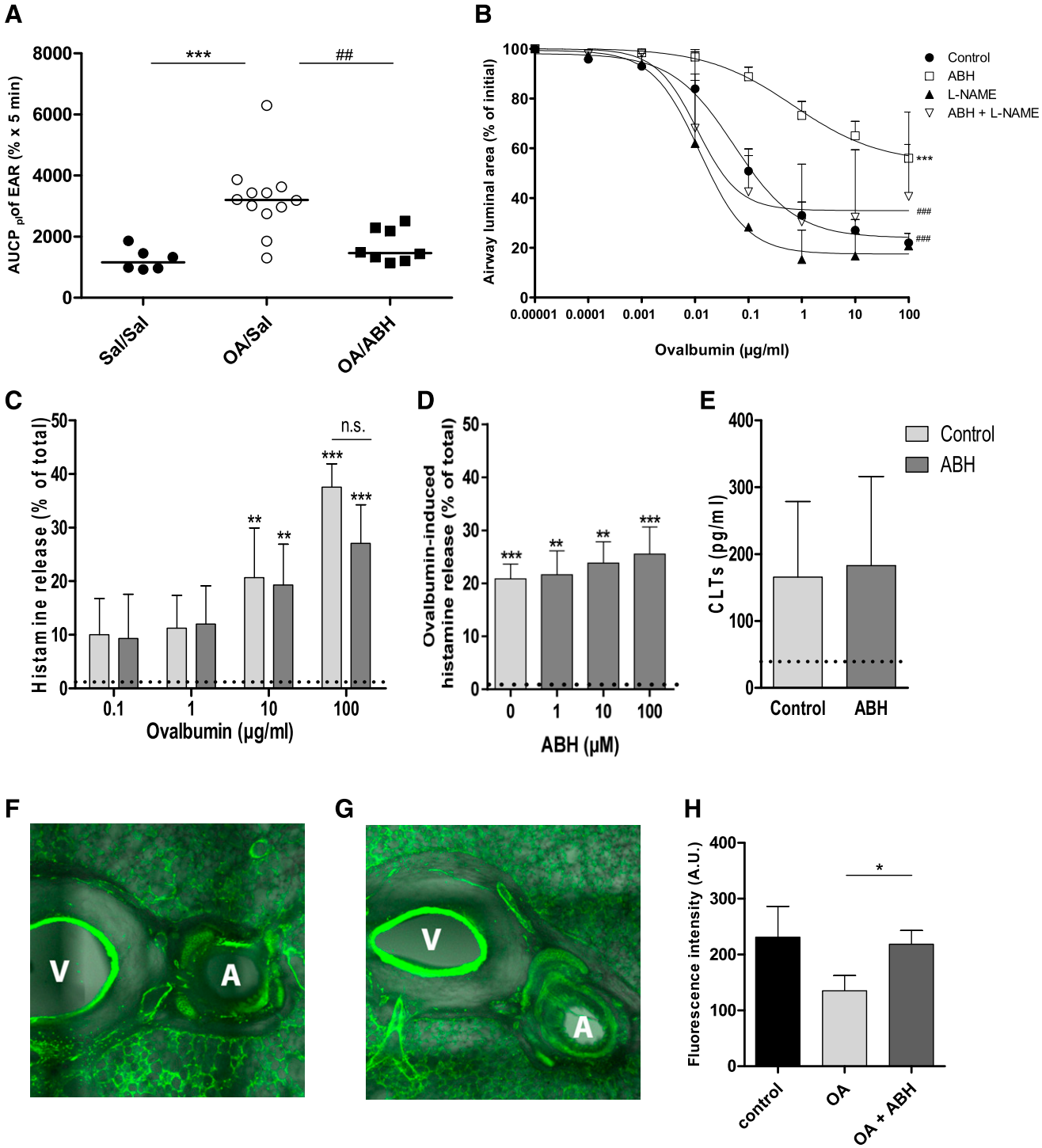


**Fig. 1.** Arginase inhibition affects allergen-induced airway narrowing in allergic guinea pigs. (A) Effects of inhaled saline or ABH on allergen (OA)-induced EAR. A group of saline-treated, saline-challenged guinea pigs serves as control. The magnitude of the EAR is presented as area under the $P_{pl}$ 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 with saline-challenged/saline-treated (Sal/Sal) animals, $^{\#\#}P < 0.01$ compared with 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 PCLSs of OA-sensitized guinea pigs. Statistical difference of the means was determined by two-way ANOVA with Newman-Keuls post hoc test. Data represent means ± S.E.M. of five animals. $^{***}P < 0.001$ compared with control. $^{\#\#\#}P < 0.001$ compared with ABH. (C) OA (0.1, 1, 10, or 100 μM)-induced histamine release of untreated and ABH (1 μM)-pretreated PCLSs of OA-sensitized guinea pigs. Histamine release was calculated as percentage 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 represent means ± S.E.M. of five animals. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with spontaneous histamine release (dotted line). (D) OA (10 μM)-induced histamine release of untreated PCLSs and PCLSs pretreated with different concentrations of ABH (1, 10, 100 μM). Data represent means ± S.E.M. of six animals. (E) OA (10 μM)-induced CLT release of untreated and ABH-pretreated PCLSs 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 represent means ± S.E.M. of five animals. Visualization of NO production in the airways in response to OA challenge (10 μM) in (F) untreated and (G) ABH (1 μM)-pretreated guinea pig PCLSs using the fluorescent probe DAF-2 DA. Produced

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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 $IC_{50}$ values were calculated. ABH and Mars' compound are both able to completely inhibit urea production by arginase 1 (Fig. 2A) and arginase 2 (Fig. 2B) in a dose-dependent manner. Moreover, Mars' compound had slightly lower $IC_{50}$ values for both arginase isoenzymes than ABH.

Next, PCLSs 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 (Fig. 2C). This was associated with a decreased maximal effect ($E_{max}$) to OA by 1 and 10 μM ABH ($P < 0.001$) and a decreased sensitivity ($EC_{50}$) to OA by all three concentrations ($P < 0.05$) compared with control. Pretreatment of PCLSs with Mars' compound (1 μM) resulted in a significant inhibition of airway narrowing ($P < 0.001$) and increased $EC_{50}$ ($P < 0.05$) compared with control, without affecting the $E_{max}$ (Fig. 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 guineas pig PCLSs 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 $IC_{50}$ 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 PCLSs 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 Fig. 4, A and B, the racemic compound and its enantiomers had similar $IC_{50}$ values for the in vitro inhibition of both arginase 1 and 2. The racemic SHK081-5 protected against maximal OA-induced airway narrowing in PCLSs in a concentration-dependent manner (Fig. 4C), which was significant after pretreatment with 1, 10, and 100 μM SHK081-5 ($P < 0.001$) compared with control. The enantiomers SHK277 and SHK278 also reduced allergen-induced airway narrowing (Fig. 4, D and E). Interestingly, these compounds were already able to induce significant protection on OA-induced airway narrowing at 0.01 μM and significantly decreased $E_{max}$ to OA at 0.1 ($P < 0.05$), 1, and 10 μM SHK277 ($P < 0.01$) and 0.1 and 1 μM SHK278 ($P < 0.001$) compared with control. Furthermore, pretreatment with 0.1, 1, and 10 μM SHK278 significantly increased $EC_{50}$ values for OA compared with control.

The in vitro and ex vivo efficacies of the two enantiomers of SHK186-6, SHK242 and SHK243, are shown in Fig. 5. SHK242 completely inhibited rhArg1 and rhArg2, with $IC_{50}$ values of 3.4 and 2.9 μM, respectively. By contrast, the other enantiomer, SHK243, hardly inhibited rhArg1 and only partially inhibited rhArg2 and showed $IC_{50}$ values of 36.4 μM for arginase 1 and 5.0 μM for arginase 2 (Fig. 5, A and B). In comparison, the racemic compound SHK186-6 showed an $IC_{50}$ value of 22.5 μM for rhArg1 (Fig. 3). Ex vivo, both enantiomers effectively suppressed OA-induced maximal airway narrowing (Fig. 5, C and D); furthermore, 10 μM SHK242 ($P < 0.05$) and 0.1 μM SHK243 ($P < 0.001$) were able to significantly increase $EC_{50}$ to OA compared with 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. 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 Fig. 6A, OA challenge resulted in the development of AHR after the EAR compared with 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 (Fig. 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 Fig. 6C, OA challenge resulted in a significant EAR compared with 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 with 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 ($P < 0.001$; Fig. 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 with saline-challenged animals. Treatment with the arginase inhibitors did not affect total inflammatory cell number or eosinophils (Supplemental Fig. 1).

## 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

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 represent means ± S.E.M. in seven animals. Statistical difference of the means was determined by Student's *t* test. *$P < 0.05$ compared with OA. A, airway; A.U., arbitrary unit; $AUCP_{pl}$, area under the pleural pressure-time curve; Sal, saline; V, vessel.Sal/Sal, saline challenged,saline treated; OA/Sal, OA challenged, saline treated; OA/AB, OA challenge, ABH treated.

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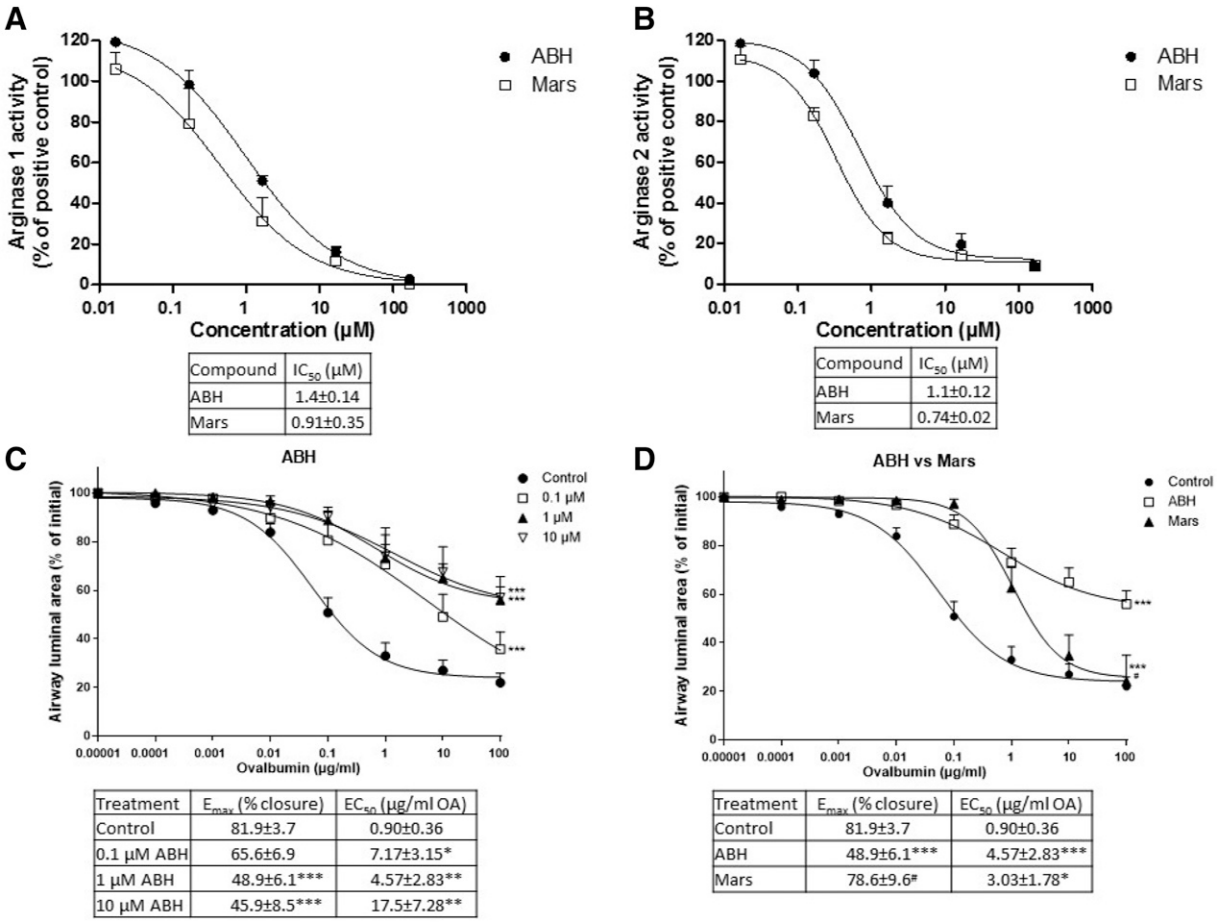



| Compound | $IC_{50}$ (μM) |
|---|---|
| ABH | 1.4±0.14 |
| Mars | 0.91±0.35 |

| Compound | $IC_{50}$ (μM) |
|---|---|
| ABH | 1.1±0.12 |
| Mars | 0.74±0.02 |

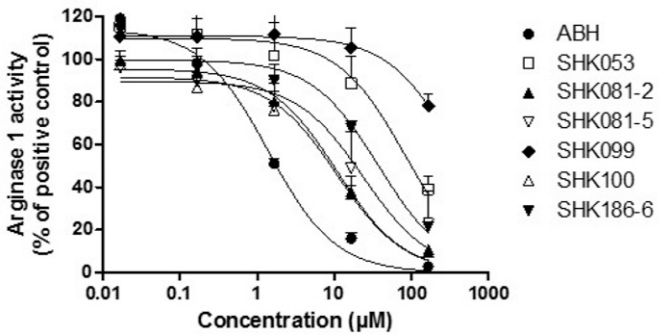
| Treatment | $E_{max}$ (% closure) | $EC_{50}$ (μg/ml OA) |
|---|---|---|
| Control | 81.9±3.7 | 0.90±0.36 |
| 0.1 μM ABH | 65.6±6.9 | 7.17±3.15* |
| 1 μM ABH | 48.9±6.1*** | 4.57±2.83** |
| 10 μM ABH | 45.9±8.5*** | 17.5±7.28** |

| Treatment | $E_{max}$ (% closure) | $EC_{50}$ (μg/ml OA) |
|---|---|---|
| Control | 81.9±3.7 | 0.90±0.36 |
| ABH | 48.9±6.1*** | 4.57±2.83*** |
| Mars | 78.6±9.6# | 3.03±1.78* |

**Fig. 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 represent means ± S.E.M. of three to five independent experiments. Ex vivo efficacy of ABH (0.1, 1, and 10 μM) (C) or ABH (1 μM) and Mars' compound (1 μM) (D) to inhibit allergen (OA)-induced airway narrowing. OA dose-response curves in untreated and arginase inhibitor–pretreated PCLSs of OA-sensitized guinea pigs. Data are presented as means ± S.E.M. of 5–18 animals. Statistical difference of the means was determined by one- or two-way ANOVA with Newman-Keuls post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control, $^{\#}P < 0.05$ compared with ABH treated.

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 necessarily translate into a high ex vivo efficacy, as (in contrast to ABH) Mars' compound did not reduce the $E_{max}$ (Fig. 2). These differences may be explained by differences in the bioavailability of these relatively hydrophilic compounds, which may inhibit effective




| Compound | $IC_{50}$ (μM) |
|---|---|
| ABH | 1.4±0.14 |
| SHK053 | 49.0±20.6 |
| SHK081-2 | 10.6±1.07 |
| SHK081-5 | 26.0±17.0 |
| SHK099 | 57.2±13.7 |
| SHK100 | 11.8±5.22 |
| SHK186-6 | 22.5±5.04 |

**Fig. 3.** In vitro efficacy of selected novel arginase inhibitors. In vitro efficacy of several novel compounds to inhibit recombinant human arginase 1. Data represent means ± S.E.M. of three to six independent experiments.

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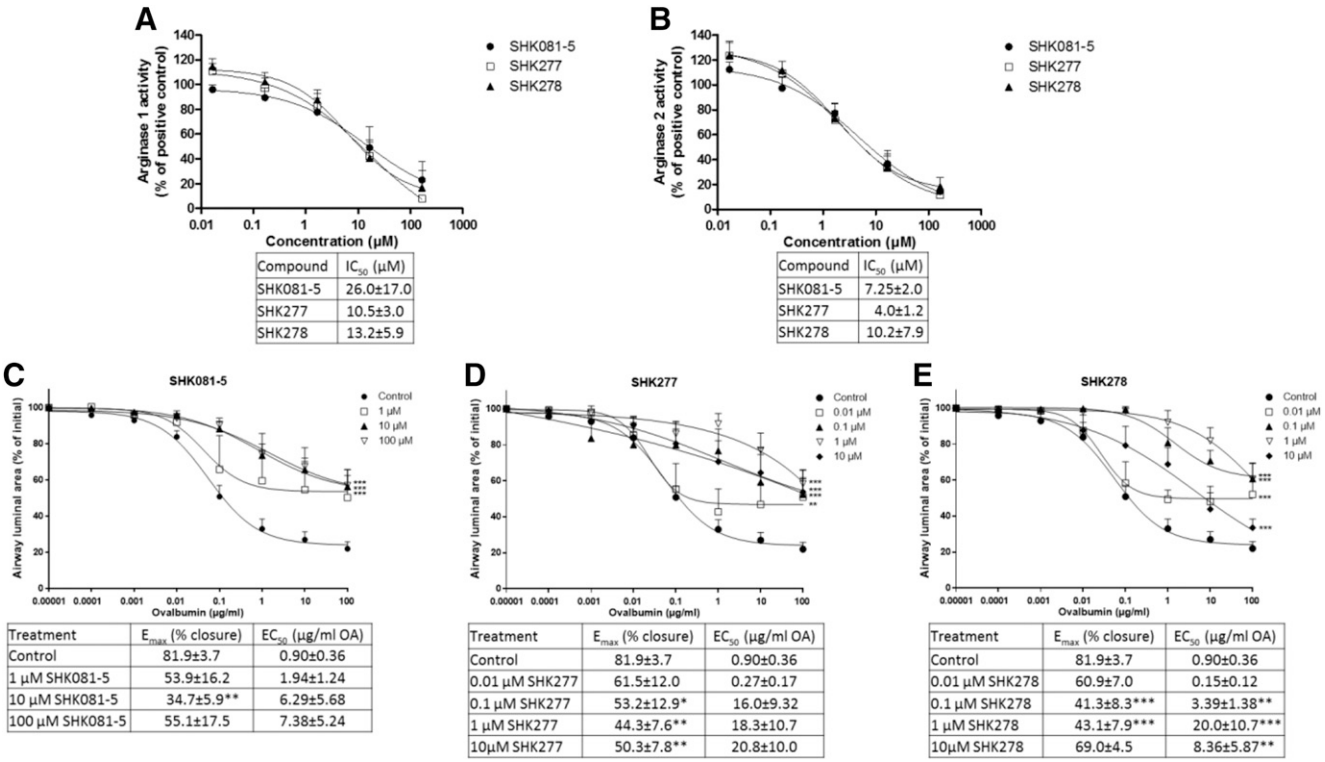


| Compound | $IC_{50}$ (μM) |
|---|---|
| SHK081-5 | 26.0±17.0 |
| SHK277 | 10.5±3.0 |
| SHK278 | 13.2±5.9 |

| Compound | $IC_{50}$ (μM) |
|---|---|
| SHK081-5 | 7.25±2.0 |
| SHK277 | 4.0±1.2 |
| SHK278 | 10.2±7.9 |

| Treatment | $E_{max}$ (% closure) | $EC_{50}$ (μg/ml OA) |
|---|---|---|
| Control | 81.9±3.7 | 0.90±0.36 |
| 1 μM SHK081-5 | 53.9±16.2 | 1.94±1.24 |
| 10 μM SHK081-5 | 34.7±5.9** | 6.29±5.68 |
| 100 μM SHK081-5 | 55.1±17.5 | 7.38±5.24 |

| Treatment | $E_{max}$ (% closure) | $EC_{50}$ (μg/ml OA) |
|---|---|---|
| Control | 81.9±3.7 | 0.90±0.36 |
| 0.01 μM SHK277 | 61.5±12.0 | 0.27±0.17 |
| 0.1 μM SHK277 | 53.2±12.9* | 16.0±9.32 |
| 1 μM SHK277 | 44.3±7.6** | 18.3±10.7 |
| 10μM SHK277 | 50.3±7.8** | 20.8±10.0 |

| Treatment | $E_{max}$ (% closure) | $EC_{50}$ (μg/ml OA) |
|---|---|---|
| Control | 81.9±3.7 | 0.90±0.36 |
| 0.01 μM SHK278 | 60.9±7.0 | 0.15±0.12 |
| 0.1 μM SHK278 | 41.3±8.3*** | 3.39±1.38** |
| 1 μM SHK278 | 43.1±7.9*** | 20.0±10.7*** |
| 10μM SHK278 | 69.0±4.5 | 8.36±5.87** |

**Fig. 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 represent means ± S.E.M. of three to six independent experiments. Ex vivo efficacy of SHK081-5 (1, 10, and 100 μM) (C), SHK277 (0.01, 0.1, 1, and 10 μM) (D), and SHK278 (0.01, 0.1, 1, and 10 μM) (E) to inhibit OA-induced airway narrowing in PCLSs of OA-sensitized guinea pigs. Data are presented as means ± S.E.M. of five to seven animals. Statistical difference of the means was determined by one- or two-way ANOVA with Newman-Keuls post hoc test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control.

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 in which 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 procontractile 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 is caused by increasing NO synthesis, thereby inhibiting mast cell degranulation. However, our present results indicate that this is unlikely because, 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 with 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; Boer et al., 1999) and patients with asthma (Silkoff et al., 2000; Ricciardolo et al., 2001, 2008), 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 an 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 coincubation with L-NAME, thus showing that arginase inhibition increases NO production. A similar mechanism was shown to underlie arginase inhibitor–induced relaxation of inhibitory nonadrenergic noncholinergic 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 (Popa et al., 1973; Muccitelli et al., 1987; Ricciardolo et al., 2008). Furthermore, we and others have shown that guinea pig PCLSs can be used as a model for both allergic asthma and COPD, as they share similar features with PCLSs obtained from patients

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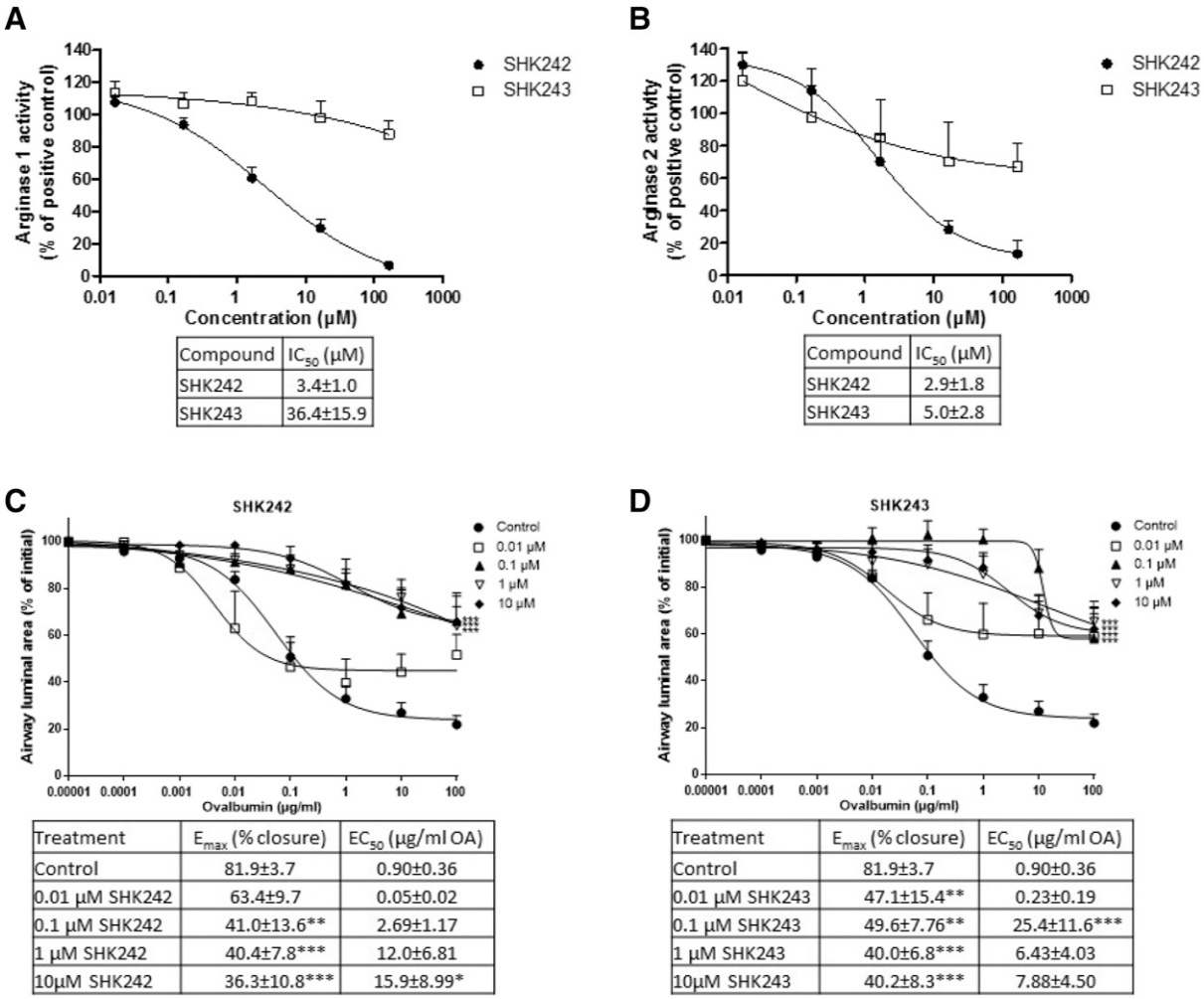


| Compound | $IC_{50}$ (μM) |
|---|---|
| SHK242 | 3.4±1.0 |
| SHK243 | 36.4±15.9 |

| Compound | $IC_{50}$ (μM) |
|---|---|
| SHK242 | 2.9±1.8 |
| SHK243 | 5.0±2.8 |

| Treatment | $E_{max}$ (% closure) | $EC_{50}$ (μg/ml OA) |
|---|---|---|
| Control | 81.9±3.7 | 0.90±0.36 |
| 0.01 μM SHK242 | 63.4±9.7 | 0.05±0.02 |
| 0.1 μM SHK242 | 41.0±13.6** | 2.69±1.17 |
| 1 μM SHK242 | 40.4±7.8*** | 12.0±6.81 |
| 10μM SHK242 | 36.3±10.8*** | 15.9±8.99* |

| Treatment | $E_{max}$ (% closure) | $EC_{50}$ (μg/ml OA) |
|---|---|---|
| Control | 81.9±3.7 | 0.90±0.36 |
| 0.01 μM SHK243 | 47.1±15.4** | 0.23±0.19 |
| 0.1 μM SHK243 | 49.6±7.76** | 25.4±11.6*** |
| 1 μM SHK243 | 40.0±6.8*** | 6.43±4.03 |
| 10μM SHK243 | 40.2±8.3*** | 7.88±4.50 |

**Fig. 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 represent means ± S.E.M. of four to six independent experiments. Ex vivo efficacy of SHK242 (0.01, 0.1, 1, and 10 μM) (C) and SHK243 (0.01, 0.1, 1, and 10 μM) (D) to inhibit OA-induced airway narrowing in PCLSs of OA-sensitized guinea pigs. Data are presented as means ± S.E.M. of five to six animals. Statistical difference of the means was determined by one- or two-way ANOVA with Newman-Keuls post hoc test. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with control.

(Ressmeyer et al., 2006; Maarsingh et al., 2019). 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, in which 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 setup 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 because 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 that 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 SHK277 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 with ABH, the chances of side effects are expected to be even lower for our novel compounds, since they have more elaborate structures that

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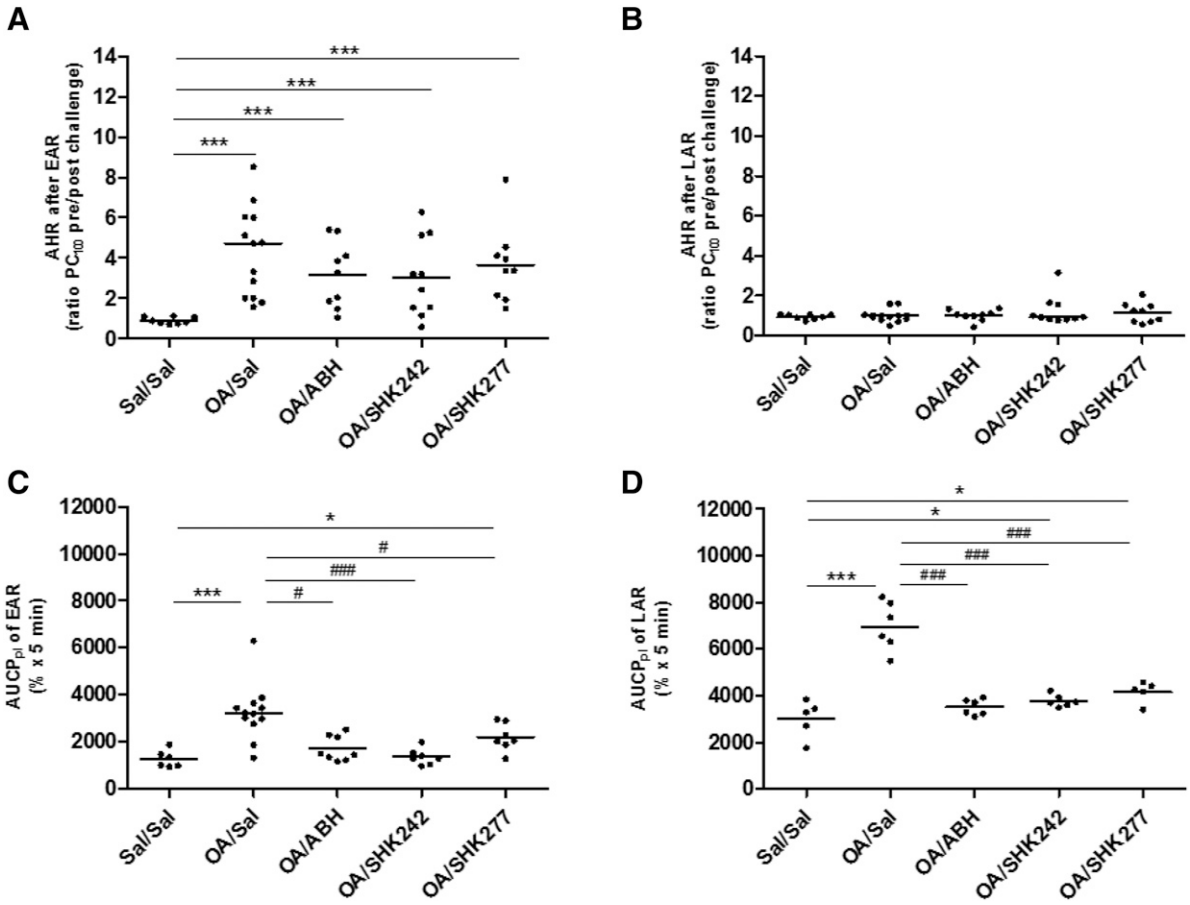


**Fig. 6.** Effect of ABH and novel arginase inhibitors on allergen-induced AHR and allergic reactions. Effects of inhaled saline, ABH, SHK242, or SHK277 (25 mM all, nebulizer concentrations, 3 minutes) on allergen (OA)-induced AHR at 6 (A) or 24 hours (B) after OA challenge. Data are expressed as the ratio of the baseline histamine $PC_{100}$ value prechallenge to the histamine $PC_{100}$ value at 6 (EAR) and 24 hours (LAR) postchallenge and are represented as medians of $n$ = 8–13 animals. Also, the effects on the magnitude of EAR (C) and LAR (D) were measured. Data are 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 with saline-challenged/saline-treated (Sal/Sal) animals. $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$ compared with OA-challenged/saline-treated (OA/Sal) animals. AUCpl, area under the pleural pressure-time curve; Sal, saline.

bind not only 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 with ABH (Morris, 2009).

Despite efforts in expanding the treatment options for patients with asthma, 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, 2009). Local and systemic changes in arginase and arginine levels are observed in patients with asthma and can be linked to, among others, asthma severity and lung function (Maarsingh et al., 2009a; 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 with 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

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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.

#### Acknowledgments

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#### Authorship Contributions

*Participated in research design:* van den Berg, Maarsingh, Kistemaker, Kema, Elsinga, Dömling, Meurs, Gosens.

*Conducted experiments:* van den Berg, Erceg, Hulsbeek, Boekema, van Faassen.

*Contributed new reagents or analytic tools:* Kurhade, Kema, Dömling.

*Performed data analysis:* van den Berg.

*Wrote or contributed to the writing of the manuscript:* van den Berg, Kurhade, Meurs, Gosens.

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