

Effect of a very late antigen-4 receptor antagonist on allergen-induced airway responses and inflammation in asthma

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Summary

Background Very late antigen-4 (VLA₄) plays a key role in the recruitment of eosinophils in allergic responses in animal studies.

Objective We investigated whether pretreatment with multiple doses of a VLA₄ receptor antagonist, HMR 1031, protects against allergen-induced airway responses and airway inflammation in humans.

Methods Fourteen asthmatics (7F/7M), 18–49 years, PC₂₀ forced expiratory volume in 1 s (FEV₁) methacholine (M) (<8 mg/mL; FEV₁ 82.3–116.1% predicted) with dual responses to inhaled allergen participated in a double-blind, placebo-controlled, cross-over study. Each treatment period consisted of 9 days, separated by ≥2 weeks. Exhaled nitric oxide (eNO), PC₂₀FEV₁(M) and hypertonic saline-induced sputum was obtained on Days 1, 7 and 9. Subjects inhaled HMR 1031 (20 mg b.i.d.) or placebo (P) on Days 1–8. On Day 8, an allergen bronchoprovocation test was performed, the airway response was measured by FEV₁, and expressed as %fall from baseline. Data from 12 evaluable subjects are presented here.

Results Both treatments were well tolerated. There was no significant difference between HMR 1031 and P in the early asthmatic response: mean AUC (0–3 h) ± SEM (%fall h): 26.01 ± 4.26 and 17.41 ± 4.26, respectively ($P = 0.18$), nor in the late response: mean AUC (3–9 h) ± SEM (%fall h): 97.09 ± 8.63 and 97.61 ± 8.63, respectively, $P = 0.97$. This corresponded to the absence of significant allergen-induced changes in PC₂₀FEV₁(M), eNO, sputum eosinophils and soluble inflammation markers between both treatment periods.

Conclusions Treatment with multiple inhaled doses of the VLA₄ antagonist, HMR 1031, did not result in detectable protection against allergen-induced airway responses or airway inflammation in asthma.

Keywords allergen bronchoprovocation test, asthma, VLA₄ receptor antagonist

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Introduction

Asthma is primarily a chronic inflammatory disease of the airways [1], which appears to correlate with airway hyper-responsiveness (AHR), a major determinant for asthma severity and control [2, 3]. According to the current hypothesis, the airway inflammation in asthma is a T-helper type 2 cell-driven process, with eosinophils as the key effector cells. Indeed, therapeutic strategies aimed at reducing the airway eosinophils resulted in a clinically significant decrease of asthma exacerbation rates, and hence a better asthma control [3]. According to modern guidelines, inhaled corticosteroids are the first choice of controller therapy [4]. However, despite their effectiveness in asthma, even high

(oral) doses of these drugs cannot completely eliminate the airway eosinophilia [5]. Hence, additional anti-inflammatory therapies targeting several aspects of the eosinophilic inflammation and their trafficking into the airways are currently being explored.

Allergen bronchoprovocation is a validated and reproducible model of inflammatory airway responses in asthma [6]. Especially, the characteristics of the late asthmatic response (LAR), in parallel with increases in airway eosinophilia and AHR, make this model an attractive tool for intervention studies when targeting the eosinophil and its pro-inflammatory mechanisms. Furthermore, validated non-invasive methodologies, such as induced sputum and measurements of nitric oxide (NO) in exhaled air, enabled the investigation of accompanying components of the airway inflammation and the effects of anti-inflammatory therapies.

Recruitment of inflammatory effector cells into the lung tissue is an important process contributing to the pathophy-

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siology of asthma. In this dynamic process, the cell–cell adhesion plays a crucial role. The $\alpha 4$ integrins, including the very late antigen-4 (VLA₄; $\alpha 4\beta 1$), are ligands expressed on the cellular surface of mononuclear leucocytes: eosinophils, basophils, lymphocytes and monocytes [7]. VLA₄ mediates cell–cell adhesion to vascular cell adhesion molecule-1 (VCAM₁) on the endothelium, and hence plays a key role in the selective recruitment of asthma-related pro-inflammatory cells (e.g. eosinophils) from the bloodstream into target tissues. Interestingly, the expression of VCAM₁ and its ligand VLA₄ has been specifically shown in bronchial biopsies of subjects with atopic asthma [8]. Therefore, blocking the VLA₄/VCAM₁ pathway may selectively inhibit the first step in the recruitment of key effector cells into target tissues [9]. Indeed, several animal studies have provided evidence that anti-VLA₄ compounds protected against allergen-induced airway responses and airway inflammation [10]. Therefore, targeting the VLA₄/VCAM₁ pathway may be an attractive tool to inhibit the eosinophilic inflammation in asthma.

HMR 1031 is a potent and selective VLA₄ receptor antagonist that selectively blocks the binding of VLA₄ to VCAM₁ and fibronectin [11]. Data from animal models of asthma showed an effective blockade of the airway response and airway inflammation following allergen challenge both in mice and in sheep (unpublished data). We tested the hypothesis that HMR 1031 inhibits the LAR to inhaled house dust mite (HDM) and reduces the associated markers of airway inflammation and hyper-responsiveness in atopic subjects with mild-to-moderate persistent asthma.

Patients and methods

Subjects

Fourteen subjects (7F/7M; 12 white, 2 black) with clinically stable, mild-to-moderate persistent asthma participated in the study (Table 1). All subjects had a history of persistent asthma for at least 1 year (according to the criteria given by Global Initiative for Asthma [4]), without any other clinically

relevant disorders. None of the subjects had smoked tobacco during the past year. In addition, subjects with a history with 10 pack-years or over were excluded. None was using concomitant anti-asthma or anti-allergy medication for at least 6 weeks prior to and during the study, except for inhaled short-acting β_2 -agonists *prn*. There was no history of viral infections of the lower airways for at least 6 weeks. Furthermore, caffeine-containing beverages and short-acting inhaled β_2 -agonists were withheld at least 12 h before each visit to the laboratory. Atopy to HDM was confirmed by a positive weal response (≥ 3 mm) to a standardized skin prick allergen kit (Soluprick, ALK-Abelló, Nieuwegein, the Netherlands). The baseline forced expiratory volume in 1 s (FEV₁) was at least 70% of predicted. AHR was demonstrated by a PC₂₀FEV₁ to methacholine less than 8 mg/mL and all subjects had a documented LAR to inhaled HDM extract at screening. The study was approved by the Ethics Committee of the Erasmus University Medical Centre Rotterdam, and all participants gave their written consent prior to the study.

Study design

The study had a randomized, double-blind, placebo-controlled, cross-over design. At least 3 weeks before the beginning of the study, all subjects were seen on three separate screening visits within 1 week, during which the inclusion criteria were examined. On screening Day 1, written informed consent was obtained, and a questionnaire, a physical examination, a blood and urine test (including a pregnancy test for females), skin prick test (SPT) (with 10 common airborne allergens, including one negative (diluent) and one positive (histamine) control) and spirometry were performed. On screening Day 2, exhaled NO (eNO) was measured, followed by a methacholine bronchoprovocation test (BPT) for the determination of the provocative concentration causing a fall in baseline FEV₁ of 20% (PC₂₀FEV₁(M)). One hour later, sputum was induced by hypertonic saline. Moreover, subjects underwent a multi-dose SPT with an HDM extract for the determination of the skin test sensitivity (SS end-point) [12]. On screening Day 3, a standardized allergen challenge test was performed with inhaled HDM extract. Subjects with a documented early asthmatic response (EAR, i.e. a fall from baseline FEV₁ $\geq 20\%$ between 0 and 3 h post-allergen) and an LAR (i.e., a fall from baseline FEV₁ $\geq 15\%$ between 3 and 9 h post-allergen) to inhaled HDM extract were enrolled in the study.

Clinically stable subjects who fulfilled all the inclusion criteria were randomized into the study. Each study period consisted of 9 consecutive days, with a washout period of at least 2 weeks. On Day 1, the inclusion criteria were verified, a physical examination was performed, followed by measurements of eNO, PC₂₀FEV₁(M), and 1 h later by sputum induction. To ensure asthma stability, the PC₂₀FEV₁(M) had to remain within one doubling concentration on Day 1 of both study periods and the mean baseline FEV₁ had to be within 10%. Moreover, stable subjects came back to the clinical unit between 19:00 and 21:00 hours, and started inhalations of the study medication (HMR 1031 20 mg or placebo, for 15 min using a handheld jet nebulizer; Pari LC Plus Universal, Starnberg, Germany). On Days 2–8, treatments were inhaled twice daily (b.i.d.) in the clinical unit at

Table 1. Subjects' characteristics at screening

Subject no.	Sex	Age (years)	FEV ₁ (% predicted)	PC ₂₀ FEV ₁ (M) (mg/mL)	PC ₂₀ HDM* (BU/dose)
0001	M	49	92.0	1.47	437.50
0002	F	18	82.3	0.48	957.0
0003	M	23	91.0	0.28	109.38
0004	M	39	89.2	2.03	375.00
0005	F	47	102.8	0.81	468.75
0006	M	22	82.4	0.82	109.38
0007	M	40	90.7	4.78	1937.50
0008	F	21	116.1	3.64	1875.00
0009	M	20	85.0	0.44	218.75
0010	F	45	95.1	0.18	242.19
0011	F	25	108.5	0.25	437.50
0012	F	28	99.2	1.68	937.50
0013	F	32	82.9	0.15	109.38
0014	M	27	92.2	0.77	187.50

*PC₂₀ HDM cumulative dose of inhaled house dust mite extract causing the early (and late) asthmatic response on screening Day 3.

FEV₁, forced expiratory volume in 1 s; (M), methacholine; F, female; M, male.

	Screening 3 days	Washout 14 days	Treatment period 1 9 days	Washout ≥14 days	Treatment period 2 9 days	End of study 10 days
			HMR 1031 8 days (20 mg bid) → Placebo			
			Placebo → HMR 1031 8 days (20 mg bid)			
PC ₂₀ M	↑		↑ ↑ ↑		↑ ↑ ↑	
eNO	↑		↑ ↑ ↑		↑ ↑ ↑	
Sputum	↑		↑ ↑ ↑		↑ ↑ ↑	
Allergen challenge	↑		↑		↑	

Fig. 1. Study design.

about the same time ± 2 h. On Days 1 and 9, study medication was inhaled only once (in the evening and in the morning, respectively). On Day 8, the allergen BPT was conducted approximately 45 min post-dosing, and the airway response was recorded for up to 9 h post-allergen. The same doses of inhaled HDM extract were used in both study periods. Blood samples for pharmacokinetics were collected from a canula in the forearm at predefined time-points (10, 20, 30, 40, 60 min, and hourly at 2–7 and 24 h post-dose). To determine the effects on allergen-induced changes in the inflammatory markers, eNO, PC₂₀FEV₁(M) and sputum induction were performed on Days 7 and 9. Throughout the study, subjects recorded any symptoms and signs of asthma, medication use and adverse events in a diary. All BPTs were performed at the same time of the day ± 2 h in all subjects during the study. A follow-up visit was scheduled within 10 days after the last study medication to check for occurrence of adverse events, and to perform a physical examination and a spirometry (Fig. 1).

Study medication

HMR 1031 (MDL 819,767)-(S)-3((S)-2-(4,4-Dimethyl-3-(4-(3-(2-methylphenyl)-ureido)-benzyl)-2,5-dioximidazoindin-1-yl)-2-(2-methylpropyl)-acetylamin)-3-phenyl-propionic acid is a low-molecular-weight VLA₄ receptor antagonist, developed by Aventis Pharmaceuticals Inc. (Bridgewater, NJ, USA) as a potential anti-inflammatory therapy for asthma. This compound was produced as a sterile aqueous solution (10 mg/mL in NaCl 0.9%) for nebulization by a commercially available jet-nebulizer (Pari LC Plus Universal). Using this nebulizer, the inhalation efficiency appeared to be 70–78% following 15 min of inhalation (respirable fraction is around 18%).

Dosing rationale

HMR 1031 has a high receptor-binding affinity and selectively inhibits the VLA₄ binding to VCAM₁ and fibronectin. The IC₅₀ for inhibition of VLA₄/VCAM₁ and

VLA₄/fibronectin binding *in vitro* was 0.7 and 0.3 nM, respectively.

The dose and dosing frequency of HMR 1031 (20 mg b.i.d.) in the current study were based on an effectivity study in sensitized sheep combined with a safety and tolerability study in 48 corticosteroid-free asthmatics in whom multiple increasing doses (5, 10 and 20 mg b.i.d.) for 7 days had previously been shown to be safe and tolerable. In this study, a linear increase in plasma concentrations, AUC and C_{max} values was noted with increasing doses of HMR 1031. Following the 20 mg b.i.d. dosing regimen for 7 days, there was a mean systemic exposure (AUC_{inf}) of 79.27 ng h/mL, a peak plasma concentration (C_{max}) of 15.59 ng/mL and a plasma half-life (T_{1/2}) of 13.58 h. Moreover, the systemic exposure appeared to be higher than the *in vitro* IC₅₀, and the functional T cell immunity tests in PBMC showed a trend towards a decrease, hence suggesting both good systemic bioavailability and potential effectivity.

Inhalation challenges and other procedures

Methacholine BPT The methacholine challenges were performed by the tidal breathing method according to a standardized protocol [13]. Serial doubling concentrations of methacholine bromide (M; 0.04–9.8 mg/mL, equals 0.15–40 µmol/mL; Janssen Pharmaceutical, Beerse, Belgium) in normal saline were prepared in the Hospital's Pharmacy, stored at 4°C and administered at room temperature. Methacholine aerosols were generated by a DeVilbiss 646 nebulizer (output 0.13 mL/min) connected to an in-and expiratory valve box with an expiratory aerosol filter (Pall Ultipor BB50T, Medica BV, Den Bosch, the Netherlands). Each concentration of M was inhaled through the mouth with the nose clipped, for 2 min, at 5 min intervals, until a $\geq 20\%$ fall in FEV₁ from post-diluent baseline was reached or the highest concentration was given. After the test, subjects received salbutamol 2 $\times$ 100 µg through an aerochamber (Volumatic®).

Allergen BPT

The allergen BPT was performed according to Cockcroft's method [12–14]. A purified aqueous allergen extract of *Dermatophagoides Pterinysinus* (SQ503, ALK-BPT, ALK-Abelló), with 0.5% phenol as a preservative, was used for the BPT. Each subject had her/his own stock solution of 5 mL, containing 10 000 BU/mL, which was diluted to 25 mL with an ALK BPT diluent (phosphate-buffered saline (PBS) with 0.03% human serum albumin and 0.5% phenol), and divided into five equal portions of 5 mL containing 2000 BU/mL, stored at 4°C. Immediately before inhalation, doubling dilutions from this stock were prepared ranging from 2000 to 15.63 BU/mL. According to Cockcroft's formula, PC₂₀ allergen (APC₂₀) was predicted from PC₂₀FEV₁(M) and skin test sensitivity (SS point) derived from the SPT with diluted HDM extract (SQ503, Soluprick, ALK-Abelló) [12]. The allergen aerosols were generated by a DeVilbiss 646 nebulizer (output 0.13 mL/min) connected to an in-and expiratory valve box (Hans-Rudolph, Kansas City, MO, USA) with an expiratory aerosol filter (Pall Ultipor BB50T, Medica BV). Subjects first inhaled the allergen diluent, and provided the subsequent fall in FEV₁ remained <10%, the allergen solutions could be inhaled. Starting three doubling concentrations below the APC₂₀ predicted, 3 mL of serial dilutions of the HDM extract were aerosolized and inhaled by tidal breathing for 2 min with the nose clipped at approximately 12 min intervals, until a ≥20% fall in FEV₁ from post-diluent baseline was reached or the highest concentration was given. After testing, subjects received salbutamol ≥2 × 100 µg through an aerochamber, and the FEV₁ was measured and had to return within 10% from baseline. Each subject inhaled the same allergen concentrations during both study periods.

Response measurements

The airway responses to the inhaled aerosols were measured by FEV₁ according to standardized lung function techniques [15], and were recorded by a spirometer connected to a PC (Masterscreen FRC, Jaeger, Würzburg, Germany). Before each test, the FEV₁ was measured in triplicate, and the mean of the two highest, technically satisfactory measurements, was considered as baseline FEV₁. In the case of a BPT, the post-diluent baseline was considered as the baseline.

The airway response to methacholine was obtained by FEV₁ measurements at 30 and 90 s following each dose inhalation, and the lowest, technically satisfactory FEV₁ was implicated in the analysis. In case the second value turned out to be the lowest, a third recording was performed (at 180 s), and the lowest value was included in the calculation of the %fall from (post-diluent) baseline. The tests were discontinued once a ≥20% fall in FEV₁ from baseline was reached or the highest dose was administered.

The airway response to allergen was measured in duplicate 10 min after each dose inhalation of allergen, and repeated at 10, 20, 30, 45, 60 min, and then 2, 3, 4, 4.5; 5, 5.5; 6, 6.5; 7, 7.5; 8, 8.5; and 9 h after the inhalation that caused a fall in FEV₁ ≥20% from post-diluent baseline (i.e. EAR). At each time-point, the highest, technically satisfactory FEV₁ value was implicated in the analysis and expressed as %fall from post-diluent baseline. Measurements were discontinued be-

fore 9 h post-allergen in case any of the subjects developed clinically significant airway narrowing (either a drop in FEV₁ ≥55% from baseline or an FEV₁ <1.4 L) or experienced unbearable discomfort. In case of an emergency, rescue medication could be instituted.

Diary cards

Day- and night-time asthma symptoms scores, short-acting β₂-agonist usage, concomitant medication, potential adverse events, and pre-bronchodilator PEF measurements (three times daily) were recorded in diary cards throughout the study [16].

eNO measurements

eNO measurements were performed using a chemiluminescence analyser (Sievers NOA 280, Sievers, Boulder, CO, USA; detectable range from <0.1 to 500 000 parts per billion (p.p.b.)) according to a standardized procedure [17]. During the measurements, subjects inhaled 'NO-free' air (<2 p.p.b.). Subjects were asked to perform a slow vital capacity manoeuvre three times at a constant expiratory flow of 50 mL/s into a tube with an in-line flow resistor (5 cm H₂O/L/s, Hans-Rudolph) using online visual monitoring. eNO was sampled continuously (response time 0.2 s) from the centre of the flow at a sample flow of 440 mL/min. The expiratory flow was measured by a Lilly-type pneumotachograph (Masterlab Jaeger), positioned downstream of the resistor. eNO concentrations were measured at a 10 s plateau and expressed as p.p.b. Successive recordings were made, and the mean of three satisfactory measurements within 5% was applied in the analysis.

Sputum induction

Sputum was induced by a previously validated protocol [18]. Hypertonic saline aerosols (NaCl 4.5%) were generated at room temperature by a DeVilbiss Ultraneb 2000 ultrasonic nebulizer with a calibrated particle size (mass median aerodynamic diameter 4.5 µm) at maximal output (2.5 mL/min). The aerosols were administered to the subjects through a 100 cm long tube with an internal diameter of 22 mm, and were inhaled through the mouth via a two-way valve (No. 2700, Hans-Rudolph), with the nose clipped. Before inhalation of the aerosols, baseline FEV₁ was recorded and, for safety reasons, 200 µg salbutamol was administered through a metered dose inhaler connected to an aerosol chamber (Volumatic[®], GlaxoSmithKline, Zeist, The Netherlands). Subsequently, the subjects inhaled hypertonic saline aerosols three to four times at 5 min intervals with the nose clipped. After each inhalation, they were asked to blow their nose, to rinse their mouth and throat with water and to expectorate sputum into a plastic container by coughing. For safety, the FEV₁ was recorded after every 5 min of inhalation; sputum induction was discontinued after ±15 min of inhalation time, or whenever the FEV₁ fell by >20% from baseline. After testing, an additional dose of salbutamol was administered, when needed.

Sputum processing

Whole-sputum samples were processed according to a validated protocol [18, 19]. Briefly, the volume of the induced sputum samples was determined and mixed with an equal volume of dithiothreitol 0.1% (Sputolysin, Calbiochem, La Jolla, CA, USA). To ensure adequate homogenization, the samples were placed in a shaking water bath at 37 °C for 15 min, interrupted once by gently mixing the sample with a plastic pipette. Subsequently, the homogenized sputum samples were centrifuged at 350 g for 10 min at room temperature. The supernatants were aspirated and frozen at -80 °C, until analysis. The cell pellets were resuspended in PBS, followed by filtration through a nylon mesh (pore size 48 µm) to remove clumps. Cell counts and assessments of viability were performed in a haemocytometer (Tamson, Zoetermeer, the Netherlands), in order to obtain an estimate of the total number of cells per sample. Subsequently, the sample was diluted with PBS to a final concentration of $\pm 0.5 \times 10^6$ cells/mL, which was used for preparation of cytocentrifuge slides (350 g, 3 min, 50 µL/cytospin) (Cytofuge, Nordic Immunological Labs BV, the Netherlands). Differential cell counts of eosinophils, neutrophils, lymphocytes, macrophages, bronchial epithelial cells and squamous cells were performed on May-Grünwald-Giemsa stained, coded cytopsins by a qualified cytopathologist. In addition, mast cells were counted on Toluidine blue-stained cytopsins. To correct for the variable salivary contamination, differential leucocyte and cylindric epithelial cell counts were expressed as a percentage of 500 nucleated cells excluding squamous cells. For each sample, differential cell counts were performed twice by the same observer, and the mean data were used in the analysis. If >80% of the cells consisted of squamous cells, the quality of the sputum sample was judged as unsatisfactory and was excluded from analysis.

In the sputum supernatant, levels of albumin (nephelometric assay), fibrinogen and tryptase were determined according to 'standard operating procedures' at the Hospital Laboratory of Clinical Chemistry. IL5, IL-8 and TNF- α were determined by ELISAs (BioSource, Etten-Leur, the Netherlands; detection limits <4, <5, and 1.7 pg/mL, respectively). Concentrations of eosinophil cationic protein (ECP) were measured using a fluoroimmunoassay (FEIA; Pharmacia CAP System ECP FEIA, Kabi Pharmacia Diagnostics AB, Uppsala, Sweden; detection limit: 2 µg/L).

Data analysis

The PC₂₀FEV₁(M) was calculated by linear interpolation of the airway responses just below and just above 20% fall in FEV₁ [13].

The airway responses to inhaled allergen were expressed as percentage fall in FEV₁ from the post-diluent baseline, and were plotted as time-response curves during both study periods. The activity of HMR 1031 on the allergen-induced airway responses was determined by comparing the corresponding area under the time-response curve (AUC) for both the EAR (0–3 h post-allergen), and the LAR (3–9 h post-allergen) between HMR 1031 and placebo. The trapezoidal rule was applied for the calculation of the AUCs [13]. Similarly, the differences in the maximal percent fall in FEV₁

during both time intervals were compared between the two treatments. In addition, the differences in allergen-induced changes in eNO, methacholine PC₂₀ and the sputum differential cell counts (mast cells, eosinophils, neutrophils, lymphocytes, macrophages, epithelial and squamous cells) and soluble markers (albumin, fibrinogen, tryptase, ECP, TNF- α , IL-5, IL-8) were assessed by comparing the corresponding values 24 h before and 24 h after allergen challenge between the two treatments.

For the assessment of treatment differences (HMR 1031 vs. placebo) in the outcome parameters, an ANOVA appropriate to the two-period, two-sequence, two-treatment cross-over design was used. The ANOVA model contained factors of treatment, sequence and subject within sequence. Carry-over effects were examined for the primary analysis variables. *P*-values <0.05 were considered statistically significant.

The sample size of 12 evaluable subjects for this study was based upon the simplifying assumption for a comparison of the two treatments using a paired *t*-test. Given this assumption, the calculated sample size required to detect a 30% mean difference in the LAR was 12 subjects ($\alpha = 0.05$ (two tailed), $\beta = 0.10$ (one tailed), power = 90%; within-subject standard deviation = 25%) [20].

Results

Safety

All 14 subjects completed the study, and HMR 1031 was generally well tolerated. Two adverse events were considered to be possibly or probably HMR 1031 related: i.e. nausea and pharyngolaryngeal pain occurring in subject 0002 and 0004, respectively. There were no clinically significant changes in physical examination, serum haematology, chemistry, urine or spirometry.

Spirometry data

Spirometry data for subjects 0001 and 0014 have been excluded from analysis: subject 0001 became clinically unstable during period II and hence did not receive the final dose of allergen extract on Day 8. Subject 0014 also became unstable during period II. Hence, for the results with respect to diaries, airway calibre, allergen-induced airway responses, airway responsiveness to methacholine and airway inflammation, data of only subjects 0002–0013 were included.

Pharmacokinetics

Pharmacokinetic data were collected on Day 8. Subject 0002 was excluded from analysis because of inappropriate sample collections. HMR 1031 (20 mg) was rapidly absorbed with a median *T*_{max} of 0.67 h. Plasma concentrations followed a bi-exponential decline after reaching their peak. The mean $\pm$ SD plasma AUC_{inf} and *C*_{max} values were 52.10 $\pm$ 22.53 ng h/mL and 10.28 $\pm$ 3.11 ng/mL, respectively. The mean *T*_{1/2} for HMR 1031 was 3.63 $\pm$ 1.03 h (Fig. 2). Compared with a previous study, the estimation of the mean terminal half-life in this study was lower. The difference in the half-lives may be a result of the different collection times (7 vs. 24 h in the previous study).

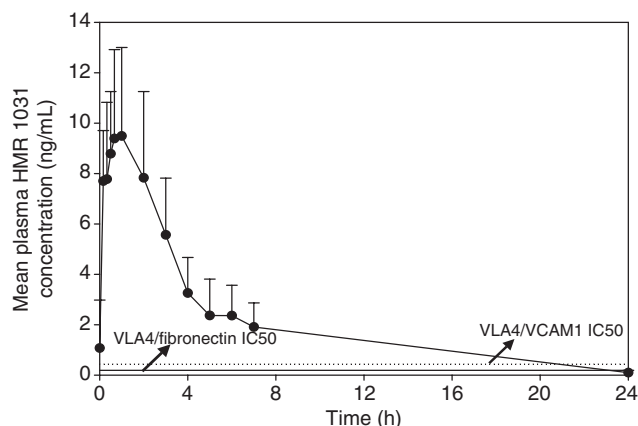


Fig. 2. Mean ($\pm$ SD) plasma concentration–time profile of HMR 1031 following a single inhaled 20 mg dose (Day 8) by patients with mild-to-moderate persistent asthma (data obtained from $n = 11$ subjects).

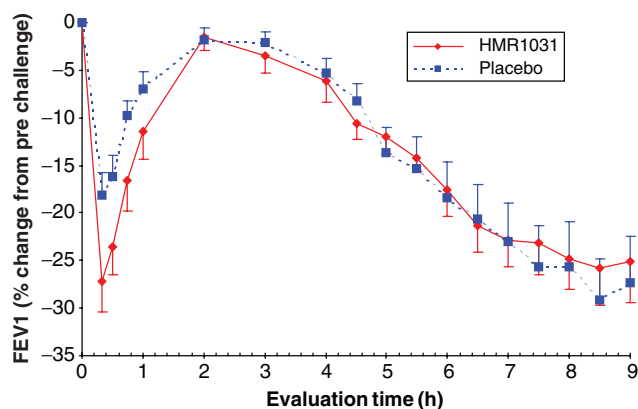


Fig. 3. Allergen-induced airway responses as mean ($\pm$ SEM) from 0 to 9 h post-allergen during both HMR 1031 and placebo study periods. There were no differences in early asthmatic response and late asthmatic response between both treatment periods.

Airway calibre

HMR 1031 did not affect FEV₁, and pre-allergen mean baseline FEV₁ was not significantly different on both Days 8 (mean $\pm$ SEM: 3.59 ± 0.034 L (HMR 1031); 3.65 ± 0.034 L (P); $P = 0.21$). At 24 h post-challenge, baseline FEV₁ was not significantly reduced following both treatments, as compared with pre-allergen baseline (mean difference $\pm$ SEM: -0.35 ± 0.07 L, $P = 0.46$ (HMR 1031), and -0.44 ± 0.07 L ($P = 0.05$ (P)). Neither was there a significant difference between these changes in baseline FEV₁ between the two treatments ($P = 0.39$).

Allergen-induced airway responses

Inhaled HDM extract induced dual airway responses in all subjects. There were no significant differences in the magnitude of the EAR between HMR 1031 and P, both in terms of AUC (0–3 h) and maximal %fall from baseline FEV₁ (AUC (0–3 h) as %fall h: 26.01 ± 4.26 (HMR 1031) and 17.41 ± 4.26 (P) ($P = 0.18$); and max%fall: -27.47 ± 3.05 (HMR 1031) and -18.20 ± 3.05 (P) ($P = 0.05$)). Neither were there any significant differences between the treatments in these parameters of the LAR (AUC (3–9 h): 97.09 ± 8.63 (HMR 1031) and 97.61 ± 8.63 (P), %fall h ($P = 0.97$); and

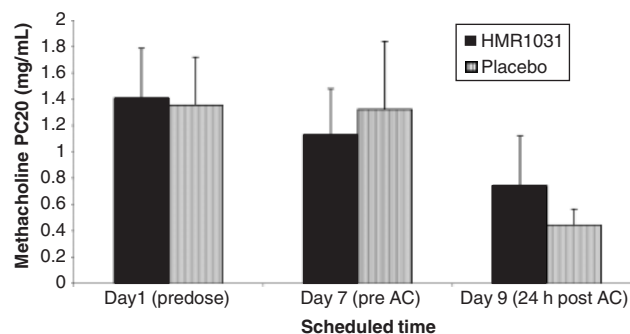


Fig. 4. Airway hyper-responsiveness as mean ($\pm$ SEM) PC₂₀FEV₁(M) on Days 1, 7 and 9 of both HMR 1031 and placebo study periods. There were no significant differences (in the changes) in PC₂₀FEV₁(M) between the treatment periods. FEV₁, forced expiratory volume in 1 s; (M), methacholine.

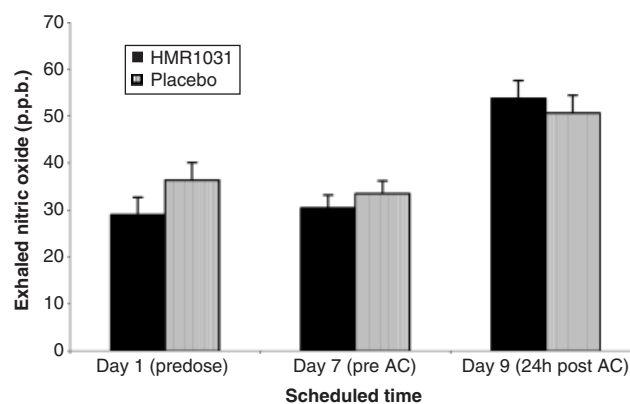


Fig. 5. Exhaled nitric oxide (eNO) as mean ($\pm$ SEM) eNO on Days 1, 7 and 9 of both HMR 1031 and placebo study periods. There were no significant differences in the changes in eNO between the treatment periods.

max %fall: -28.62 ± 1.80 (HMR 1031) and -31.75 ± 1.80 (P) ($P = 0.24$)) (Fig. 3). Analysis showed no statistically significant carry-over effects of the treatments.

Allergen-induced changes in airway responsiveness to methacholine

There was a trend towards an increase in allergen-induced airway responsiveness to methacholine after placebo (mean change in PC₂₀ $\pm$ SEM: -0.95 ± 0.43 mg/mL doubling concentrations, $P = 0.05$) but not after HMR 1031 (-0.36 ± 0.47 doubling concentrations, $P = 0.46$). However, these allergen-induced changes in airway responsiveness to methacholine were not significantly different between the two treatments ($P = 0.37$) (Fig. 4).

Allergen-induced changes in eNO

The allergen challenge induced a significant increase in eNO following both HMR 1031 and placebo treatment ($P > 0.0001$). In accordance with the lack of effect of HMR 1031 on the airway responses, the changes in eNO were not significantly different between the two treatments (mean change $\pm$ SEM: 22.94 ± 2.74 p.p.b. (HMR 1031), and 17.11 ± 2.49 p.p.b. (P), $P = 0.14$) (Fig. 5).

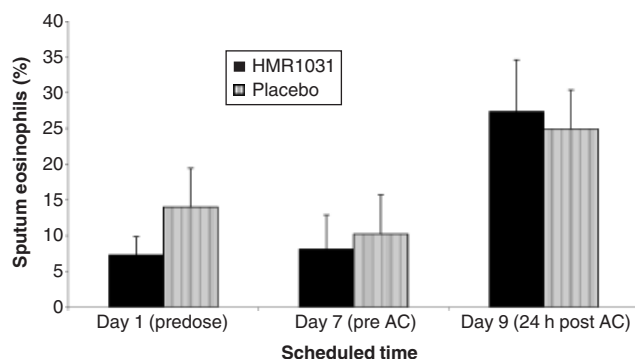


Fig. 6. Sputum eosinophils as mean ($\pm$ SEM) % sputum eosinophils on Days 1, 7 and 9 of both HMR 1031 and placebo treatment periods. There were no significant differences in the changes in % sputum eosinophils between the treatment periods.

Allergen-induced changes in sputum cell differentials

Adequate sputum samples (<80% squamous cells) were gathered from all subjects on all indicated days. Pre-allergen, there was no significant difference in the % sputum eosinophils between both study periods (mean $\pm$ SEM: $7.34 \pm 2.90\%$ (HMR 1031) and $14.05 \pm 2.90\%$ (P), $P = 0.13$). The allergen challenge induced a significant increase in the % sputum eosinophils (mean increase $\pm$ SEM: $19.78 \pm 6.60\%$, $P = 0.01$ (HMR 1031), and 15.20 ± 6.60 , $P = 0.04$ (P), respectively). However, there was no significant difference in the allergen-induced increases in % sputum eosinophils between HMR 1031 and placebo ($P = 0.63$) (Fig. 6).

Allergen-induced changes in soluble markers

In agreement with the other results, as compared with placebo, HMR 1031 did not significantly affect any of the soluble markers studied (data not shown).

Discussion

The findings of the present study show that treatment with the inhaled VLA₄ receptor antagonist, HMR 1031, 20 mg b.i.d. for 8 days, neither reduces the allergen-induced airway eosinophilia nor any other relevant markers of airway inflammation, and consequently does not protect against allergen-induced airway responses in subjects with atopic asthma.

In an allergen model in mice, a small-molecule VLA₄ receptor antagonist effectively blocked the allergen-induced airway eosinophilia and substantially reduced the eosinophil activation products, following intranasal administration [21]. Similarly, treatment with another aerosolized anti-VLA₄ compound effectively prevented allergen-induced airway responses (early and late allergen-induced airway response and hyper-responsiveness to carbachol at 24 h post-allergen) and airway eosinophilia in sensitized sheep [22]. In addition, administration of similar doses of inhaled HMR 1031 in sensitized mice (<1.0 mg/kg) and sheep (0.1 mg/kg) resulted in a substantial reduction of the allergen-induced airway inflammatory response and airway responses, respectively (Aventis, unpublished data). However, despite these protective properties and reductions in inflammation parameters in

animals, suggesting that drugs targeting VLA₄ indeed can protect against allergen-induced airway eosinophilia and the associated airway responses, we were not able to reproduce these data in asthmatic patients *in vivo*.

We believe that we used appropriate methodology and study conditions to demonstrate clinical proof of the mechanism for VLA₄ antagonists in asthma. First, we used validated and reproducible methods in clinically stable and steroid-free asthmatics [13, 14, 17, 18]. All evaluable subjects, while on placebo treatment, showed a LAR accompanied by rises in the non-invasive parameters of inflammation following allergen challenge. Moreover, we considered that based on previous repeatability studies [19] in subjects with similar asthma characteristics, a sample size of 12 subjects would enable us to detect a difference of 4.9% in the changes in sputum eosinophils between the two pretreatments ($\alpha = 0.05$ (two-tailed), $\beta = 0.10$ (one-tailed), power = 90%). Second, we based our treatment regimen on a previous study in subjects with similar asthma characteristics, in whom treatment with inhaled corticosteroids (budesonide 400 μ g/day) for 7 days resulted in a significant reduction of the allergen-induced airway responses (EAR, LAR, AHR at 24 h post-allergen), together with the associated eosinophilia and activation markers [20]. Third, we applied the same dosing regime and nebulizer that have previously provided a good lung bioavailability in non-asthmatic subjects [23]. In this study, a scintigraphic evaluation following inhalation of HMR 1031 (20 mg) showed that approximately 18% was deposited in the airways, corresponding to approximately 3.6 mg of the initial dose, indicating good lung bioavailability [23]. In our study, there appeared to be a good systemic exposure reflected by the high plasma peak concentrations (16 nM), far exceeding the *in vitro* IC₅₀ of VLA₄/VCAM₁ (0.7 nM), which, given the drug's poor oral bioavailability combined with the above-mentioned data, presumably reflects the absorption from the pulmonary tract and hence underscores previous observations. And finally, we used the same inhaled dose of HMR 1031 that has effectively blocked the LAR and airway eosinophilia in sensitized mice and sheep (Aventis, unpublished data). Assuming a similar inhaled fraction for both species, the equivalent dose for humans would be approximately 7 mg (0.1 mg/kg for 70 kg). In our study, subjects inhaled 40 mg/day and hence, given the airway deposition of 18% of the initial dose, this should be sufficient to cause similar effects as in the sheep. Despite these data, the lack of effect of HMR 1031 in our study may still be ascribed to a low bioavailability locally at the receptor site since plasma concentrations may not fully reflect the situation within the airways.

How can we explain the lack of effect of HMR 1031 in the present study, despite the effectiveness of anti-VLA₄ compounds previously shown in animal models of allergic inflammation? An explanation for our findings could be that different, as yet unidentified, mechanisms may prevail in the migration of eosinophils into human airways *in vivo* than those mediated by the interaction of VLA₄/VCAM₁ [24]. Indeed, evidence has been provided for an alternative migration mechanism for eosinophils from both animal and human studies [25–27]. Grayson and colleagues exposed eosinophils from asthmatic subjects to various pro-inflammatory mediators and cytokines. Following exposure, some of these substances appeared to up-regulate the expression of

the β_2 -integrins on the surface of the eosinophils. Furthermore, this integrin has been shown to act as an alternative ligand for VCAM₁ [25, 26]. These data have been confirmed and extended in a rabbit model of asthma [27]. In this model, it was shown that eosinophil recruitment to the lung along with their activation are mediated by a combination of VLA₄, a β_1 -integrin and a lymphocyte functional antigen-1 (LFA-1; CD18/CD11a): a β_2 -integrin. Moreover, treatment of the rabbits with an anti-CD11a monoclonal antibody (moAb) resulted in a significantly greater reduction in eosinophil numbers in the bronchoalveolar lavage than treatment with anti-CD49d (VLA₄) moAb, indicating a major contribution of the β_2 -integrin to the recruitment of eosinophils to the lung [27].

In conclusion, treatment with multiple inhaled doses of the VLA₄ receptor antagonist, HMR 1031, did not offer any protection against allergen-induced airway responses or any clinically relevant reduction in the associated markers of airway inflammation in subjects with mild-to-moderate persistent asthma. Despite application of drug doses and dosing schemes that have previously shown anti-inflammatory potential in animals and adequate exposure in asthma, this study showed negative findings. These findings suggest that the VLA₄/VCAM₁ complex may not play a predominant role in the recruitment of eosinophils into asthmatic airways following inhalation of allergen. Hence, evaluation of the exact role of the VLA₄/VCAM₁ complex in the migration of eosinophils to target sites in response to pro-inflammatory stimuli in asthma warrants further investigation.

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