

Original article

Inhaled vs subcutaneous effects of a dual IL-4/IL-13 antagonist in a monkey model of asthma

Background: Pitrakinra is a recombinant protein derived from human interleukin-4 (IL-4) that binds to IL-4R α and acts as a competitive antagonist of IL-4 and IL-13. The studies reported here compare the dose-ranging effects of pitrakinra on allergen-induced airway hyperresponsiveness (AHR) and airway eosinophilia when administered subcutaneously (s.c.) or by inhalation to the *Ascaris suum*-sensitive cynomolgus monkey for the purpose of elucidating the primary site of pitrakinra's anti-asthmatic action.

Methods: Airway responsiveness to inhaled methacholine and bronchoalveolar lavage cell composition was determined before and after three allergen exposures with a 1-week course of twice-daily (b.i.d.) s.c. or inhaled pitrakinra or placebo treatment.

Results: Treatment with s.c. pitrakinra significantly reduced allergen-induced AHR, with a maximum effect of a 2.8- to 3.8-fold increase in methacholine PC₁₀₀ relative to control ($P < 0.05$) observed at b.i.d. s.c. doses of 0.05–0.5 mg/kg. Inhaled pitrakinra also significantly reduced AHR with a similar maximum effect of a 2.8- to 3.2-fold increase in methacholine PC₁₀₀ relative to control ($P < 0.05$) at nominal b.i.d. doses of 3–100 mg. The maximal effect on AHR following inhalation was observed at a plasma concentration which exhibited no efficacy via the subcutaneous route. The effect of pitrakinra on lung eosinophilia was not statistically significant following either route of administration, although lung eosinophil count was reduced in all studies relative to control.

Conclusion: Local administration of pitrakinra to the lung is sufficient to inhibit AHR, one of the cardinal features of asthma, indicating the therapeutic potential of inhaled pitrakinra in the treatment of atopic asthma.

A. Tomkinson¹, J. Tepper¹,
M. Morton¹, A. Bowden¹, L. Stevens¹,
P. Harris², D. Lindell³, N. Fitch⁴,
R. Gundel⁵, E. B. Getz⁶

¹Aerovance Inc., Berkeley, CA, USA; ²Pfizer, PGRD, Groton, CT, USA; ³Pfizer, PGRD, New London, CT, USA; ⁴Sanofi-Aventis, Bridgewater, NJ, USA; ⁵Elusys Therapeutics Inc., Pine Brook, NJ, USA; ⁶Oriel Therapeutics Inc., Berkeley, CA, USA

Key words: airway hyperresponsiveness; air inflammation; asthma; IL-4; IL-4 receptor alpha; IL-13.

A. Tomkinson
Aerovance Inc.
2929 Seventh Street
Berkeley
CA 94710
USA

Accepted for publication 19 June 2009

Despite the heterogeneity of asthma, clinical and genetic studies imply that both interleukin-4 (IL-4) and interleukin-13 (IL-13) are central to the pathogenesis of atopic asthma (1–4).

Both cytokines have demonstrated multiple biological functions considered crucial to the development of asthma. In particular, IL-4 is critical for the commitment of T cells to the CD4 + Th2 phenotype, whilst both cytokines regulate IgE isotype switching in B cells (5–7). Both cytokines have several other relevant functions including enhancement of antigen presentation, recruitment of inflammatory cells via VCAM-1 upregulation, exacerbation of airway smooth muscle contractility and induction of airway mucus secretion (8–14).

Further evidence for the role of IL-4 and IL-13 in asthma comes from *in vivo* experimental studies. Administration or overexpression of either cytokine in the airways can induce airway eosinophilia, mucus production, and airway hyperresponsiveness (AHR) (13, 15–18). Use of inhibitors or mice deficient in either cytokine confirms the principal role of IL-4 in the development of antigen responsiveness, whilst IL-13 appears more important during subsequent allergen provocation (17, 19–25). Despite the apparent dominant role of IL-13 during the effector phase, IL-4 is capable of inducing the allergic phenotype in the absence of IL-13. Thus a therapeutic strategy targeting both IL-4 and IL-13 may be beneficial (26).

Pitrakinra (also known as AER 001 or BAY 16-9996) is a human IL-4 variant (R121D/Y124D) that prevents formation of both the IL-4 activated type I receptor heterodimer (IL-4R α chain/common γ -chain), and the IL-4/IL-13 activated type II receptor heterodimer (IL-13R α 1 chain/IL-4R α chain), and thus antagonizes both human IL-4- and IL-13-induced responses (27–29).

Abbreviations: AHR, airway hyperresponsiveness; ANCOVA, analysis of covariance; AUC, area under curve; BAL, bronchoalveolar lavage; GM-CSF, granulocyte-macrophage colony stimulating factor; IL-4/13, interleukin-4/13; MCh, methacholine; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; s.c., subcutaneous.

In accord with its expected mechanism of action, pitrakinra has demonstrated a protective effect on the allergen-induced late phase asthmatic response in two independent, randomized, double-blind, placebo-controlled, parallel group phase 2a clinical trials (30). In these clinical trials, inhalation dosing produced a significant effect despite low systemic exposure relative to subcutaneous (s.c.) injection, suggesting the lung to be the primary site of pitrakinra's anti-asthmatic action.

The studies described here were undertaken to further clarify the primary site of pitrakinra's anti-asthmatic action. In these studies, the dose-ranging efficacy of pitrakinra on allergen-induced AHR and airway eosinophilia was investigated following both s.c. and inhalation routes of administration to the *Ascaris suum*-sensitive cynomolgus monkey. Secondly, we investigated the effect of s.c. pitrakinra on the acute wheal and flare response to injected allergen to provide further insight into the potential therapeutic effects of longer term treatment. Finally, we report here the *in vitro* results of pitrakinra's ability to separately antagonize the proliferative response of TF-1 cells to IL-4 and IL-13.

Methods

TF-1 proliferation assay

Functional antagonistic activity was confirmed against the proliferative response of TF-1 cells (erythroleukemia cell line) to human (h) IL-4 or human (h) IL-13 (R&D Systems, Minneapolis, MN, USA).

T-lymphocyte proliferation

Antagonist activity against human and monkey IL-4 was examined using the proliferative response of phytohemagglutinin (PHA) blasts prepared from peripheral blood mononuclear cells (PBMC) from heparinized human and cynomolgus monkey blood incubated for 72 h with either human or monkey IL-4 $\pm$ pitrakinra. Proliferation was assessed by ^3H -thymidine incorporation.

Animals

Experiments were performed in *A. suum* sensitive, adult (6–11 kg) male cynomolgus monkeys (*Macaca fascicularis*) in compliance with the Institutional Animal Care and Use Committee. All procedures, except s.c. administration of pitrakinra, were performed in intubated animals under anesthesia (Ketamine 7 mg/kg; Xylazine 1.2 mg/kg, i.m.).

Cutaneous wheal and flare response

Intradermal injections (40 μl) of PBS, *A. suum* extract (1 : 10 000 of stock solution) or histamine dihydrochloride (0.025 mg/ml) were administered to each animal's shaved chest every other week (weeks 0–12). Pitrakinra (1 mg/kg, $3 \times$ /week, s.c.) or PBS was administered weeks 2 through 12 to four animals per treatment group in a parallel group design. The area of the wheal and flare and a severity score [1, 1.5 or 2] was measured immediately after and again approximately

15 min after each allergen injection and an overall score (area in $\text{mm}^2 \times$ severity score) assigned for the change in response.

Allergen-induced AHR and airway eosinophilia

Experimental protocols for allergen challenge, pitrakinra administration and response assessment are shown in Fig. 1. These studies employed a cross-over design in which an active treatment period was bracketed by vehicle control periods. Active or control periods were separated by a minimum of 6 weeks. *Ascaris suum* solution (1 : 1 stock dilution) was aerosolized using a Bird micronebulizer and Mark 7A respirator (Viasys Health Care, Yorba Linda, CA, USA) delivering 15 breaths/min for 2 min. AHR to aerosolized methacholine (MCh; 0.1–100 mg/ml) was determined in anesthetized ventilated animals. The data were reported as the dose of MCh required to produce a 100% increase in baseline lung resistance (PC_{100}). Bronchoscopic bronchoalveolar lavage (BAL) (saline, 15 ml) was performed after measurement of AHR to obtain total leukocyte and differential cell counts using standard methods.

Administration of pitrakinra

To evaluate the effects of pitrakinra on allergen-induced AHR, four experiments using s.c. delivery (0.005, 0.025, 0.05 and 0.5 mg/kg) and four experiments using inhalation delivery (nominal dose 0.5, 1, 3 and 100 mg) were performed. A single experiment to evaluate the effects of pitrakinra (1 mg/kg) on the cutaneous wheal and flare was performed. Both s.c. and inhaled dose levels were selected to cover the anticipated clinical dose range. For s.c. administration, pitrakinra was diluted to achieve a dose volume of 200 μl /kg for each dose level. For inhalation studies, pitrakinra was administered (nominal dose volume 3 ml) as an aerosol using a Bird Mark 7A Respirator and the Aeroneb Pro nebulizer (Aerogen Ltd., Galway, Ireland). Approximately 4% of the nominal dose was delivered to the monkey lung as measured by *in vitro* analysis and *in vivo* gamma scintigraphy.

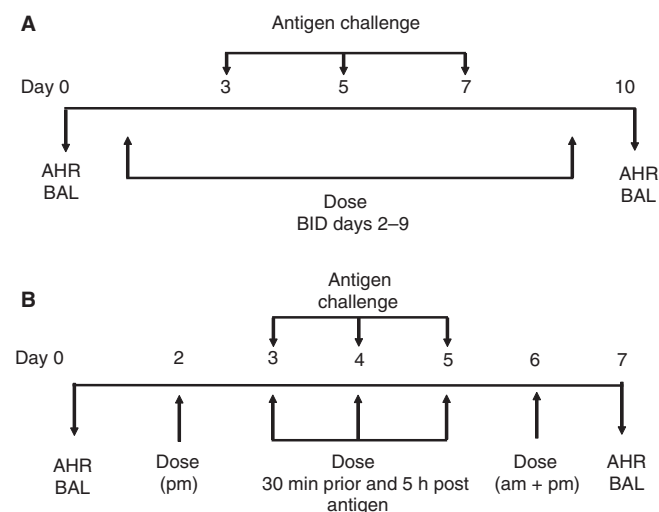


Figure 1. Primate asthma models. (A) Subcutaneous study: Airway responsiveness to MCh and BAL were determined before (day 0) and 3 days after (day 10) allergen exposure. Pitrakinra was administered b.i.d., days 2–9. (B) Inhalation studies: Airway responsiveness to MCh and BAL were determined before (day 0) and 2 days after (day 7) allergen exposure. Pitrakinra was administered on day 2, and b.i.d. days 3–6.

Blood sampling and assays

Blood samples were drawn from the femoral vein for pharmacokinetic analysis. Pitrakinra in K₂EDTA plasma was measured by ELISA at Aerovance, Inc. The pitrakinra ELISA used commercially available anti-hIL-4 capture and detection monoclonal antibodies (BD Biosciences) with a lower limit of quantitation of 0.5 ng/ml.

Pharmacokinetic analysis

PK data following inhalation were analyzed using a noncompartmental approach (WinNonlin Pro version 4.1, Pharsight Corporation, St. Louis, MO, USA) to identify area under the concentration-*vs*-time curve within the dosing interval ($AUC_{0-\tau}$) for each individual. Average plasma concentration (C_{avg}) within the dosing interval (τ) was calculated as the geometric mean $AUC_{0-\tau}/\tau$. No PK data were collected from the s.c. pitrakinra injection studies. Instead, a nonlinear mixed effects model (NONMEM VI 2.0 GloboMax, ICON Development Solutions, Redwood City, CA, USA) with PLT Tools (PLTsoft, San Francisco, CA, USA) was fit to PK data collected following intravenous ($n = 5$) and s.c. injection ($n = 34$) of 0.025–3.0 mg/kg pitrakinra to cynomolgus monkeys, across three independent studies. $AUC_{0-\tau}$ was derived from this model for each of the s.c. dose cohorts.

Statistical analysis

The effect of pitrakinra on AHR was interpreted using ANCOVA, with baseline PC_{100} as covariate and animal identification as a factor. PC_{100} values were log-transformed prior to analysis. Estimated treatment geometric means and treatment ratio with 95% CI and associated *P*-value were determined. The effect of pitrakinra on post-treatment airway eosinophilia was interpreted following log transformation of eosinophil count per ml BAL fluid and included a factor for animal identification. To avoid multiple comparisons arising from statistical comparison of the two treatment groups at each observation time point, the time course of the wheal and flare response was summarized for each animal by the area under the response-*vs*-time curve using linear trapezoidal integration. The resulting area values were interpreted using ANCOVA with treatment group as the primary variable of interest and a covariate for the baseline wheal and flare value.

Results

Pitrakinra inhibits human IL-4 and human IL-13 induced TF-1 cell proliferation

Pitrakinra inhibited hIL-4 (0.033 nM)-stimulated TF-1 proliferation with an IC_{50} (concentration that inhibits activity by 50%) of 0.38 ± 0.25 nM ($n = 20$) and hIL-13 (0.416 nM)-stimulated TF-1 proliferation with an IC_{50} of 1.28 ± 1.59 nM ($n = 7$). Pitrakinra had no independent agonistic activity.

Pitrakinra inhibits IL-4 induced proliferation of human and monkey T lymphocytes

Species cross-reactivity of hIL-4 and hence pitrakinra is limited, restricting the options for animal studies to

nonhuman primates. Thus the ability of pitrakinra to antagonize both human and monkey lymphocyte proliferation to both hIL-4 and monkIL-4 was examined. In the presence of increasing concentrations of pitrakinra, the stimulatory activities of hIL-4 and monkIL-4 were increasingly inhibited to background levels. IC_{50} values for pitrakinra vs hIL-4 (0.67 nM) and monkIL-4 (0.67 nM) on human lymphocytes were 4.49 ± 1.17 ($n = 4$) and 3.18 ± 0.92 ($n = 4$), respectively, and for pitrakinra vs hIL-4 and monkIL-4 on monkey lymphocytes were 3.69 ± 1.37 ($n = 7$) and 2.10 ± 1.12 ($n = 7$), respectively.

Effects of subcutaneous pitrakinra on AHR and airway eosinophilia

Subcutaneously administered pitrakinra conferred a 2.8-fold (95% CI 1.6–4.9, $P = 0.0049$) and 3.8-fold (95% CI 1.6–9.1, $P = 0.0115$) reduction in AHR (as assessed by PC_{100}) relative to control treatment at doses of 0.5 and 0.05 mg/kg b.i.d., respectively (Fig. 3). Pitrakinra at 25 μ g/kg b.i.d. caused a 2.1-fold reduction relative to control on AHR but the effect was not significant (95% CI 0.7–5.9, $P = 0.1015$). There was no observed difference between pitrakinra and control treatment at the lowest dose tested, 5 μ g/kg b.i.d. (0.3-fold reduction relative to control, 95% CI 0.1–1.4, $P = 0.1165$). Results are presented in Table 1.

Pitrakinra did not consistently produce a statistically significant reduction in lung eosinophil count, although each of the dose cohorts trended toward an improvement on pitrakinra relative to control (Table 2).

Effects of inhaled pitrakinra on AHR and airway eosinophilia

We tested the hypothesis that the predominant site of pitrakinra effect on AHR was local by delivering pitrakinra via inhalation in the prophylactic model previously used to test s.c. injected drug. Results are presented in Tables 1 (AHR) and 2 (airway eosinophilia).

Pitrakinra conferred a 2.8-fold (95% CI 1.6–4.9, $P = 0.0020$) and 3.2-fold (95% CI 1.2–8.8, $P = 0.0279$) reduction in AHR relative to control at nebulizer ('nominal') doses of 100 and 3 mg b.i.d. respectively. There was no statistically significant difference between pitrakinra and control treatment at the 1-mg b.i.d. nominal dose. However, the effect of a 0.5-mg b.i.d. nominal dose on AHR was significant (2.4-fold reduction relative to control, 95% CI 1.0–5.7, $P = 0.0546$) (Fig. 2). These studies demonstrate that inhaled pitrakinra effectively reduces allergen-induced AHR.

As with s.c. injection, the effect of pitrakinra on airway eosinophil count did not achieve consistent statistical significance, but did uniformly favor pitrakinra over control. To investigate whether the absence of statistical significance was a type II error resulting from small numbers of observations, eosinophil count data were

Table 1. Effect of pitrakinra on allergen-induced airway hyperresponsiveness as measured by the provocative concentration of methacholine required to cause a doubling of airway resistance.

Prophylactic effect of pitrakinra on airway hyperresponsiveness								
Route	Dose	Comparison	Pitrakinra log ₁₀ [PC ₁₀₀] (SD)	Control log ₁₀ [PC ₁₀₀] (SD)	Ratio	95% CI	Two-sided <i>P</i> value	No of subjects
s.c.	0.5 mg/kg b.i.d.	Pitrakinra/control	0.646 (0.496)	0.345 (0.581)	2.76	1.56, 4.89	0.005	4
s.c.	0.05 mg/kg b.i.d.	Pitrakinra/control	0.811 (0.526)	0.169 (0.284)	3.78	1.57, 9.10	0.012	4
s.c.	25 µg/kg b.i.d.	Pitrakinra/control	1.094 (0.190)	0.793 (0.431)	2.05	0.71, 5.93	0.102	4
s.c.	5 µg/kg b.i.d.	Pitrakinra/control	0.451 (0.986)	0.883 (0.380)	0.34	0.08, 1.44	0.117	4
inh.	100 mg b.i.d.	Pitrakinra/control	0.807 (0.790)	0.430 (0.643)	2.76	1.55, 4.91	0.002	7*
inh.	3 mg b.i.d.	Pitrakinra/control	1.414 (0.604)	1.049 (0.518)	3.23	1.18, 8.83	0.028	5†
inh.	1 mg b.i.d.	Pitrakinra/control	1.371 (0.804)	1.239 (0.788)	1.42	0.53, 3.80	0.446	6
inh.	0.5 mg b.i.d.	Pitrakinra/control	1.227 (0.767)	0.906 (0.647)	2.36	0.98, 5.71	0.055	6

Inhaled doses listed indicate the amount loaded into the nebulizer. Post-treatment log₁₀(PC₁₀₀) values are listed for each cohort as mean (SD). The treatment ratio with corresponding 95% confidence interval and *P* value reflects ANCOVA-adjusted geometric mean post-treatment PC₁₀₀.

*Baseline lung resistance not collected from one of eight animals due to difficulties with anesthesia induction.

†Post-treatment PC₁₀₀ not collected from one of six animals; ventilator problems caused early termination of the methacholine response.

Table 2. Effect of pitrakinra on allergen-induced airway eosinophilia as measured by total eosinophil count per ml of bronchoalveolar lavage fluid

Prophylactic effect of pitrakinra on airway eosinophilia								
Route	Dose	Comparison	Pitrakinra Eos (SD)	Control Eos (SD)	Ratio	95% CI	Two-sided <i>P</i> value	No of subjects
s.c.	0.5 mg/kg b.i.d.	Control/pitrakinra	5.057 (0.295)	5.423 (0.531)	2.32	0.71, 7.66	0.1288	3*
s.c.	0.05 mg/kg b.i.d.	Control/pitrakinra	5.675 (0.213)	5.853 (0.240)	1.52	0.90, 2.57	0.0965	4
s.c.	25 µg/kg b.i.d.	Control/pitrakinra	5.258 (0.282)	5.516 (0.178)	1.81	1.06, 3.09	0.0386	4
s.c.	5 µg/kg b.i.d.	Control/pitrakinra	6.231 (0.108)	6.319 (0.226)	1.22	0.72, 2.09	0.3140	4
inh.	100 mg b.i.d.	Control/pitrakinra	5.403 (0.214)	5.413 (0.268)	1.04	0.75, 1.44	0.8195	8
inh.	3 mg b.i.d.	Control/pitrakinra	4.990 (0.246)	5.387 (0.352)	2.50	1.40, 4.45	0.0051	6
inh.	1 mg b.i.d.	Control/pitrakinra	5.278 (0.640)	5.434 (0.418)	1.43	0.53, 3.88	0.4425	6
inh.	0.5 mg b.i.d.	Control/pitrakinra	5.269 (0.571)	5.358 (0.316)	1.23	0.64, 2.36	0.5053	6

Post-treatment log₁₀(eosinophil count) values are listed for each cohort as mean (SD). The treatment ratio with corresponding 95% confidence interval and *P* value reflects adjusted geometric mean post-treatment eosinophil count.

*Post-treatment BAL not collected from one of four animals due to insufficient depth of anesthesia.

analyzed collectively, with the sole exclusion of the 5 µg/kg b.i.d. s.c. cohort for reason of lack of a demonstrated effect on AHR at this dose level. The pooled data on eosinophil count suggest a 33% (*P* = 0.0019) reduction in lung eosinophil number on pitrakinra relative to control (pitrakinra/control ratio = 1.49, 95% CI 1.2–1.9).

Relationship between plasma concentration and effect of pitrakinra on AHR

Comparison of pitrakinra systemic exposure, as measured by plasma concentration-*vs*-time data, following inhalation *vs* s.c. administration demonstrates that inhaled pitrakinra reduces AHR at plasma concentrations insufficient for efficacy following s.c. injection (Fig. 2). The inhaled dose does not appear to achieve efficacy by way of a depot effect in which a long-lasting absorption process maintains plasma concentrations. Rather, the systemically available fraction of the inhaled dose is absorbed

rapidly followed by decline in plasma concentration with kinetics similar to those observed after s.c. injection (Fig. 3). The maximal effect on AHR (approximately a threefold reduction relative to control) appears to be similar across routes of administration.

Effect of subcutaneous pitrakinra on the acute wheal and flare response

Given the pivotal role IL-4 and IL-13 play in regulating IgE class switching of B cells, it was hypothesized that chronic pitrakinra treatment would inhibit the IgE-mediated immediate hypersensitivity reaction to allergen. The acute cutaneous response to injected allergen was significantly reduced on pitrakinra treatment as compared to placebo over the 12-week treatment period (*P* = 0.005). The maximum pitrakinra effect was observed at week 10 (8 weeks of treatment), at which time median acute responses (area in mm² × severity score [1, 1.5 or 2]) were 174 on PBS and 55 on pitrakinra,

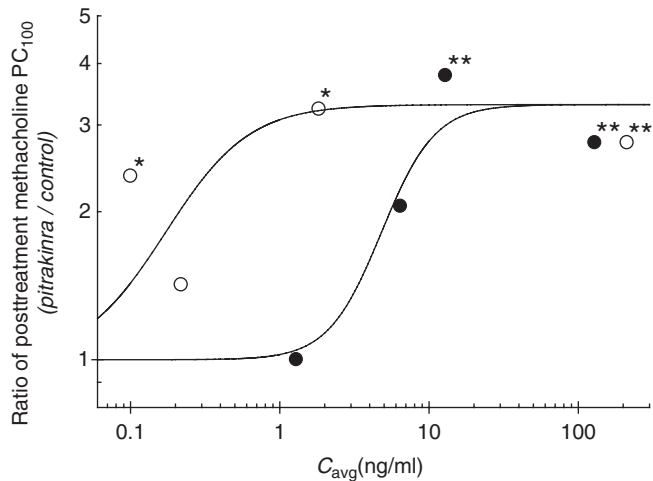


Figure 2. Dependence of the exposure-response relationship on route of administration. The relationship between average plasma concentration (C_{avg}) of pitrakinra following inhalation (O) or subcutaneous (●) administration and effect on AHR. Asterisks indicate statistical significance (* $P \leq 0.05$; ** $P \leq 0.01$) of pitrakinra effect relative to control. Efficacy values represent ANCOVA-adjusted geometric mean treatment ratios (pitrakinra/control) of post-treatment methacholine PC_{100} as listed in Table 2. Average plasma concentrations (ng/ml) were determined based on analysis of data from samples collected during efficacy studies (inhalation) and additional PK studies (s.c. injection).

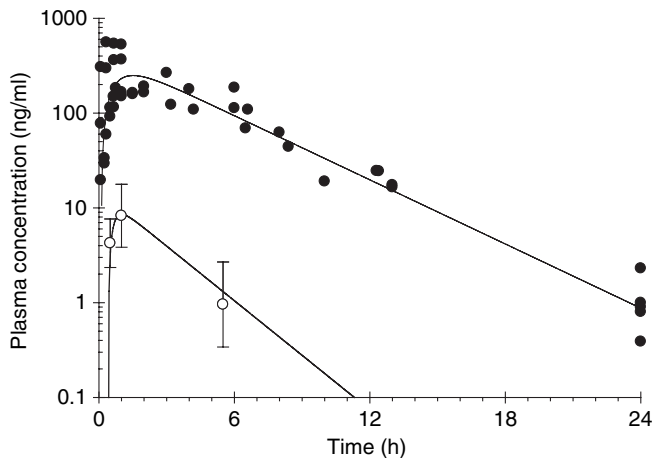


Figure 3. Concentration-vs-time profiles for pitrakinra following s.c. injection or inhalation to the cynomolgus monkey. Plasma concentration-vs-time following a 0.5-mg/kg s.c. injection (●, individual data points from five animals) or inhalation of a 3-mg nominal (device) dose (O, geometric mean of data from six animals). Geometric mean and standard deviation error bars following inhalation dosing were derived on the log-transformed scale then back-transformed to the original scale for graphical display.

representing a 68% inhibition of the hypersensitivity reaction (Fig. 4). Despite continued treatment with pitrakinra, the cutaneous wheal response returned to

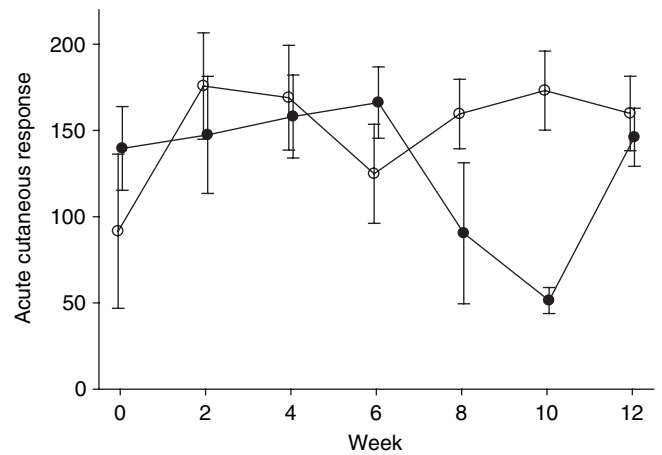


Figure 4. The effect of pitrakinra on the acute cutaneous wheal/flare response. *Ascaris suum* (i.d.) was administered every other (week 0–12). Pitrakinra (1 mg/kg) or PBS was administered by s.c. injection $3 \times$ /week for 10 weeks, beginning on week 2. Pitrakinra produced a significant reduction in the acute cutaneous response over the course of treatment relative to PBS ($P = 0.005$). (●) pitrakinra, (O) PBS, mean $\pm$ SD ($n = 4$).

pretreatment levels. Plasmon resonance analysis of plasma samples collected from treated animals at the 12-week time point demonstrated the presence of an inhibitory factor which blocked pitrakinra from binding to IL-4R α . Although the bioanalytical methodology to identify this blocking factor as an antibody was not in place at the time of this experiment, subsequent studies have confirmed development of anti-pitrakinra IgG antibodies that block binding to IL-4R α following repeated dosing to monkeys.

Discussion

Pitrakinra, a dual IL-4/IL-13 antagonist, has demonstrated a protective effect on the allergen-induced late phase response in humans with mild to moderate atopic asthma following either s.c. injection or inhalation dosing (30). In humans, inhalation dosing produced consistent efficacy despite low blood levels relative to s.c. injection. These results suggested the lung to be the primary site of pitrakinra's anti-asthmatic action, but additional data were sought to confirm the claim of a topical effect in the lung. The studies reported here provide confirmation of inhaled pitrakinra's local pharmacological activity in pulmonary tissues and support additional studies using direct administration to the lung for the treatment of asthma.

The primary site of pitrakinra's anti-asthmatic action was assessed in a nonhuman primate model of asthma in which allergen-induced AHR and airway eosinophilia, cardinal features of asthma, were measured. Using *A. suum* sensitive cynomolgus monkeys, initial studies

were performed to evaluate the prophylactic effects of subcutaneously administered pitrakinra on the induction of AHR and airway eosinophilia. Administration of pitrakinra effectively inhibited allergen-induced AHR. Inhibition of AHR was accompanied by a trend towards an effect on airway eosinophilia, as evidenced by a small reduction in BAL eosinophil number. These observations are somewhat consistent with several rodent studies. Indeed, murine IL-4 mutant proteins that bind with high affinity to the IL-4R α chain without inducing signal transduction have been developed (31, 32). Both the murine IL-4 double mutein (Q116D/Y119D) and truncated protein (C118 deletion) significantly attenuate allergen induced AHR, airway eosinophilia, mucus hyperproduction and IgE levels (15, 32).

Although pitrakinra has been shown to potentially inhibit allergen-induced AHR, the s.c. route of administration does not allow distinction between the systemic *vs* local lung contributions to the effect. To address this question and to determine if inhalation was a suitable route of administration for pitrakinra, a series of studies were performed to evaluate the effect of inhaled pitrakinra on allergen-induced AHR and airway eosinophilia. These studies demonstrated that inhaled pitrakinra effectively inhibits allergen-induced AHR with the same maximal effect achieved by s.c. administration. In the clinic, pitrakinra demonstrated a protective effect on the allergen-induced late phase response in mild to moderate atopic asthmatics following either s.c. injection or inhalation dosing (30). In both studies this was accompanied by a trend toward reduced AHR. Effects on sputum eosinophilia were only evaluated in the s.c. study, and were inconclusive due to limited samples.

Given the potential role of IL-4 and IL-13 in regulating VCAM-1 and eotaxin, the effect of the pitrakinra mouse orthologue in the murine asthma models, and the effects of IL-13 antibody in the monkey, the effects of pitrakinra on eosinophilic inflammation were less apparent than might have been expected. However, it is clear that cytokines other than IL-4 and IL-13, such as IL-5, contribute to the eosinophilic inflammatory response (33). The limited effect of pitrakinra on airway eosinophilia was independent of administration route; similar effects were observed with both s.c. and inhaled pitrakinra. Differences in the effects of pitrakinra and the mouse orthologue in the monkey and mouse, respectively, could be due to several model differences, including differences in allergen (*A. suum* *vs* *ovalbumin*), sensitization procedure (natural exposure to *A. suum* *vs* active sensitization using adjuvant), and the nature of the allergen provocation (multiple challenge to previously exposed primed airways *vs* multiple challenge to naïve airways) all of which may result in different molecular phenotypes contributing to the inflammatory component. It is possible that longer term treatment with pitrakinra would improve the effect on eosinophilic inflammation. The limited effect on eosinophilia, observed in the current study, does not necessarily

reflect a lack of effect on inflammation. Indeed, the mouse orthologues have been shown to reduce Th2 cytokine production and mucus hypersecretion in the mouse asthma model, while in the clinic, pitrakinra has been shown to reduce the inflammatory marker, fractional exhaled nitric oxide (F_ENO) (30, 32). Evaluation of airway inflammation was limited to BAL eosinophilia in the present study; it would be valuable to examine alternative markers of inflammation in future studies.

The dissociation of AHR from airway eosinophilia is not entirely surprising. The ability of pitrakinra to inhibit AHR despite limited effects on eosinophilia is supported by the direct effects of IL-4 and IL-13 on airway mucus production and airway smooth muscle responsiveness, and hence AHR (11, 34, 35). Although both nonhuman primate and rodent asthma studies utilizing antibodies to IL-5 have been able to associate inhibition of eosinophilia with inhibition of AHR, there are several rodent studies, including the use of soluble IL-4 receptor and soluble IL-13 α 2-Fc that dissociate airway eosinophilia from AHR (16, 22, 36, 37). Indeed, there is some controversy as to the role of eosinophils in the pathogenesis of AHR and asthma. Initial clinical studies utilizing antibodies against IL-5 have been negative with respect to the role of eosinophils in the disease pathogenesis, although more recent studies in asthmatics with persistent eosinophilic inflammation would suggest otherwise (38–41). These data point to the complex heterogeneous nature of the disease, and further suggest that although eosinophilia may be one of the inflammatory components of the disease it is not necessarily causative.

The dominant site of pitrakinra's action was assessed from the pharmacokinetic/pharmacodynamic (PK/PD) relationship following inhalation *vs* s.c. injection. Inhalation delivery shifted the PK/PD relationship toward lower plasma concentrations relative to s.c. injection, with maximal inhibitory effects of inhaled pitrakinra observed at a plasma concentration producing no efficacy via the s.c. route. These observations provide evidence that the primary site of pitrakinra's effect on AHR, and hence the effects of IL-4 and IL-13 during the effector phase of the allergic response to inhaled allergen, are in the lung. The observed shift in the exposure-response curve toward lower blood exposures for inhaled *vs* s.c. administration is expected to be borne out in humans as well because the dose range investigated here encompasses the clinical dose range.

Although the pharmacokinetic data and efficacy data following s.c. injection were obtained from separate studies, the overall conclusion that inhalation delivery provides efficacy at lower blood concentrations relative to s.c. injection is robust; between-animal variability in systemic exposure (AUC_{0–∞}) following s.c. injection of pitrakinra to the cynomolgus monkey is too low ($\leq 25\%$ coefficient of variation) to alter the overall conclusion. The lowest efficacy s.c. dose, 0.005 mg/kg, is lower than the lowest dose studied for PK in the cynomolgus

monkey, 0.025 mg/kg. Subcutaneous doses to humans ranging from 0.07 to 30 mg have demonstrated a statistically significant but small in magnitude increase in dose-normalized exposure with increasing dose, such that a 100-fold change in s.c. dose yields only a 55% increase in AUC. A 0.07-mg human dose is approximately equivalent (gives equal blood exposure) to a 0.0018-mg/kg dose to a 7-kg cynomolgus monkey (45), thus suggesting that in species cross-reactive to pitrakinra significant pharmacokinetic nonlinearity with dose is not expected over the monkey efficacy dose range.

Consistent with our hypothesis of local pitrakinra action, previous animal and clinical studies have demonstrated that local lung administration of IL-4 or IL-13 is sufficient to induce the asthma phenotype (15–18). Particularly, IL-13 administration to STAT6 deficient mice with STAT6 reconstituted in the epithelium alone is sufficient for AHR and mucus production (14). Moreover, both over-expression of the murine IL-4 double mutant (Q116D/Y119D) in the lung and inhaled IL-4R α antisense oligonucleotide have been successful at inhibiting allergen induced AHR and airway inflammation (15, 42).

IL-4 and IL-13 play a critical role in regulating IgE (6, 7). Although not addressed in the monkey asthma model studies, the inhibitory effect of chronic pitrakinra administration on the allergen-induced cutaneous wheal and flare (IgE-mediated immediate hypersensitivity) response was demonstrated. The effect was slow in onset (6–8 weeks) likely reflecting the time required for depletion of existing cell bound and free IgE. Whether inhibition of IgE is an important component in the monkey asthma model is unknown, although the onset of effect on the wheal and flare would suggest that it is not. Indeed, many rodent studies have demonstrated that the role of IgE in the allergen-induced lung phenotype is dependent upon the sensitization and allergen protocol used (43). In contrast, the role of IgE in asthma is more apparent since clinical studies demonstrate anti-IgE therapy can improve lung function and reduce lung inflammation to therapeutic benefit (44). Together these observations indicate a potential additional long-term therapeutic benefit of inhibiting IL-4 and IL-13 in asthma.

The results set forward here additionally provide the *in vitro* foundation to pitrakinra's hypothesized mechanism of action. Mutagenesis studies have identified the regions of IL-4 important for interaction with the IL-4R α chain, a common component of both type I and II receptors. Mutations of arginine 121, tyrosine 124 and serine 125 in the C terminus of the human protein result in IL-4 mutants that have the same capacity to bind to the IL-4R α chain as native IL-4, but have only limited or no signaling activity and are antagonists of both human IL-4- and IL-13-induced responses *in vitro* (27–29). In the present study, the IL-4 double mutant (R121D/Y124D), pitrakinra, was utilized because it had previously been shown to be the most effective IL-4 mutant antagonist of human IL-4 and IL-13 (27, 28). Using surface plasmon

resonance, the affinity of pitrakinra for the IL-4R α was demonstrated to be similar to that of human IL-4 (dissociation constant $K_d \sim 100$ pM, data not shown) confirming that the two point mutations (R121D/Y124D) did not reduce pitrakinra's affinity for the receptor (27, 29). In subsequent studies, pitrakinra potently inhibited the proliferative response of TF-1 cells to both hIL-4 and hIL-13 demonstrating the dual antagonistic nature of pitrakinra. Similar activity of pitrakinra in human and cynomolgus monkey primary T cell proliferation assays indicate the relevance of the cynomolgus monkey for our *in vivo* experiments. Additional studies, utilizing cell types considered relevant to the disease process, further confirm our observations. These include IL-4 and IL-13 induced B cell CD23 expression, epithelial cell mucus production and fibroblast proliferation, as well as IL-4-induced T cell proliferation and endothelial cell VCAM-1 expression (27, 29) (Dr. M. Wetzel, Dr. M. Longphre and T. Wong, unpublished observations).

In conclusion, the current studies demonstrate that independent of route of administration (s.c. or inhalation), the dual IL-4/IL-13 antagonist, pitrakinra, significantly reduces allergen-induced AHR in the cynomolgus monkey, with only limited inhibition of allergen-induced airway eosinophilia. The effect of pitrakinra on AHR may potentially be explained by the direct effects on the constitutive airway smooth muscle and airway epithelium. The limited effect on airway eosinophilia does not necessarily reflect lack of effect on inflammation, as eosinophilic infiltration may not be causative. Indeed effects on additional inflammatory components, as evidenced by pitrakinra's reduction of F_ENO in the clinical studies, cannot and should not be excluded, although the actual extent of pitrakinra's anti-inflammatory activities remains to be determined. Local delivery of pitrakinra to the lung not only achieves a maximal effect on AHR, but does also at plasma concentrations which exhibit little or no efficacy via the s.c. route. These observations provide evidence that the primary site of pitrakinra's anti-asthmatic action, and hence the effects of IL-4 and IL-13 during the effector phase of the allergic response to inhaled allergen, are in the lung. Taken together, these data indicate the potential of inhaled pitrakinra as a therapeutic for asthma.

Acknowledgments

The authors wish to thank Dr M. Wetzel, Dr M. Longphre and T. Wong for their assistance with completion and analysis of the TF-1 and primary cell assays.

Funding support

All work was fully supported by Bayer Healthcare and Aerovance Inc.

References

1. Kotsimbos TC, Ernst P, Hamid QA. Interleukin-13 and interleukin-4 are coexpressed in atopic asthma. *Proc Assoc Am Physicians* 1996;**108**:368–373.
2. Truyen E, Coteur L, Dilissen E, Overbergh L, Dupont LJ, Ceuppens JL et al. Evaluation of airway inflammation by quantitative Th1/Th2 cytokine mRNA measurement in sputum of asthma patients. *Thorax* 2006;**61**: 202–208.
3. Beghe B, Barton S, Rorke S, Peng Q, Sayers I, Gaunt T et al. Polymorphisms in the interleukin-4 and interleukin-4 receptor alpha chain genes confer susceptibility to asthma and atopy in a Caucasian population. *Clin Exp Allergy* 2003;**33**:1111–1117.
4. Heinzmann A, Mao XQ, Akaiwa M, Kreomer RT, Gao PS, Ohshima K et al. Genetic variants of IL-13 signalling and human asthma and atopy. *Hum Mol Genet* 2000;**9**:549–559.
5. Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G. Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 1993;**362**:245–248.
6. Finkelman FD, Katona IM, Urban JF Jr, Holmes J, Ohara J, Tung AS et al. IL-4 is required to generate and sustain in vivo IgE responses. *J Immunol* 1988;**141**:2335–2341.
7. Punnonen J, Aversa G, Cocks BG, McKenzie AN, Menon S, Zurawski G et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA* 1993;**90**:3730–3734.
8. Doyle AG, Herbein G, Montaner LJ, Minty AJ, Caput D, Ferrara P et al. Interleukin-13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-gamma. *Eur J Immunol* 1994;**24**: 1441–1445.
9. Webb DC, Cai Y, Matthaei KI, Foster PS. Comparative roles of IL-4, IL-13, and IL-4Ralpha in dendritic cell maturation and CD4+ Th2 cell function. *J Immunol* 2007;**178**:219–227.
10. Bochner BS, Klunk DA, Sterbinsky SA, Coffman RL, Schleimer RP. IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J Immunol* 1995;**154**: 799–803.
11. Kellner J, Gamarra F, Welsch U, Jorres RA, Huber RM, Bergner A. IL-13Ralpha2 reverses the effects of IL-13 and IL-4 on bronchial reactivity and acetylcholine-induced Ca²⁺ signaling. *Int Arch Allergy Immunol* 2007;**142**: 199–210.
12. Temann UA, Prasad B, Gallup MW, Basbaum C, Ho SB, Flavell RA et al. A novel role for murine IL-4 in vivo: induction of MUC5AC gene expression and mucin hypersecretion. *Am J Respir Cell Mol Biol* 1997;**16**:471–478.
13. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eosinophil production. *J Clin Invest* 1999;**103**:779–788.
14. Kuperman DA, Huang X, Nguyen L, Holscher C, Brombacher F, Erle DJ. IL-4 receptor signaling in Clara cells is required for allergen-induced mucus production. *J Immunol* 2005;**175**: 3746–3752.
15. Zavorotinskaya T, Tomkinson A, Murphy JE. Treatment of experimental asthma by long-term gene therapy directed against IL-4 and IL-13. *Mol Ther* 2003;**7**:155–162.
16. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;**282**: 2258–2261.
17. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, Rennick DM et al. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 1998;**282**:2261–2263.
18. Shi HZ, Deng JM, Xu H, Nong ZX, Xiao CQ, Liu ZM et al. Effect of inhaled interleukin-4 on airway hyperactivity in asthmatics. *Am J Respir Crit Care Med* 1998;**157**:1818–1821.
19. Coyle AJ, Le Gros G, Bertrand C, Tsuyuki S, Heusser CH, Kopf M et al. Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am J Respir Cell Mol Biol* 1995;**13**:54–59.
20. Corry DB, Folkesson HG, Warnock ML, Erle DJ, Matthay MA, Wiener-Kronish JP et al. Interleukin 4, but not interleukin 5 or eosinophils, is required in a murine model of acute airway hyperactivity. *J Exp Med* 1996;**183**:109–117.
21. Hogan SP, Mould A, Kikutani H, Ramsay AJ, Foster PS. Aeroallergen-induced eosinophilic inflammation, lung damage, and airways hyperactivity in mice can occur independently of IL-4 and allergen-specific immunoglobulins. *J Clin Invest* 1997;**99**:1329–1339.
22. Henderson WR Jr, Chi EY, Maliszewski CR. Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. *J Immunol* 2000;**164**:1086–1095.
23. Yang G, Li L, Volk A, Emmell E, Petley T, Giles-Komar J et al. Therapeutic dosing with anti-interleukin-13 monoclonal antibody inhibits asthma progression in mice. *J Pharmacol Exp Ther* 2005;**313**:8–15.
24. Kasaian MT, Donaldson DD, Tchistiakova L, Marquette K, Tan XY, Ahmed A et al. Efficacy of IL-13 neutralization in a sheep model of experimental asthma. *Am J Respir Cell Mol Biol* 2007;**36**:368–376.
25. Bree A, Schlerman FJ, Wadanoli M, Tchistiakova L, Marquette K, Tan XY et al. IL-13 blockade reduces lung inflammation after *Ascaris suum* challenge in cynomolgus monkeys. *J Allergy Clin Immunol* 2007;**119**:1251–1257.
26. Perkins C, Wills-Karp M, Finkelman FD. IL-4 induces IL-13-independent allergic airway inflammation. *J Allergy Clin Immunol* 2006;**118**:410–419.
27. Tony HP, Shen BJ, Reusch P, Sebald W. Design of human interleukin-4 antagonists inhibiting interleukin-4-dependent and interleukin-13-dependent responses in T-cells and B-cells with high efficiency. *Eur J Biochem* 1994;**225**:659–665.
28. Mueller TD, Zhang JL, Sebald W, Duschl A. Structure, binding, and antagonists in the IL-4/IL-13 receptor system. *Biochim Biophys Acta* 2002;**1592**:237–250.
29. Andrews AL, Holloway JW, Holgate ST, Davies DE. IL-4 receptor alpha is an important modulator of IL-4 and IL-13 receptor binding: implications for the development of therapeutic targets. *J Immunol* 2006;**176**:7456–7461.
30. Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet* 2007;**370**:1422–1431.

31. Grunewald SM, Kunzmann S, Schnarr B, Ezernieks J, Sebald W, Duschl A. A murine interleukin-4 antagonistic mutant protein completely inhibits interleukin-4-induced cell proliferation, differentiation, and signal transduction. *J Biol Chem* 1997;**272**:1480–1483.
32. Tomkinson A, Duez C, Cieslewicz G, Pratt JC, Joetham A, Shanafelt MC et al. A murine IL-4 receptor antagonist that inhibits IL-4- and IL-13-induced responses prevents antigen-induced airway eosinophilia and airway hyper-responsiveness. *J Immunol* 2001;**166**: 5792–5800.
33. Rosenberg HF, Phipps S, Foster PS. Eosinophil trafficking in allergy and asthma. *J Allergy Clin Immunol* 2007;**119**:1303–1310 quiz 1311–1312.
34. Eum SY, Maghni K, Tolloczko B, Eidelman DH, Martin JG. IL-13 may mediate allergen-induced hyperresponsiveness independently of IL-5 or eotaxin by effects on airway smooth muscle. *Am J Physiol Lung Cell Mol Physiol* 2005;**288**:L576–L584.
35. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, Zhu Z et al. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 2002;**8**:885–889.
36. Hamelmann E, Cieslewicz G, Schwarze J, Ishizuka T, Joetham A, Heusser C et al. Anti-interleukin 5 but not anti-IgE prevents airway inflammation and airway hyperresponsiveness. *Am J Respir Crit Care Med* 1999;**160**:934–941.
37. Mauser PJ, Pitman AM, Fernandez X, Foran SK, Adams GK 3rd, Kreutner W et al. Effects of an antibody to interleukin-5 in a monkey model of asthma. *Am J Respir Crit Care Med* 1995;**152**: 467–472.
38. Flood-Page P, Swenson C, Faiferman I, Matthews J, Williams M, Brannick L et al. A study to evaluate safety and efficacy of mepolizumab in patients with moderate persistent asthma. *Am J Respir Crit Care Med* 2007;**176**:1062–1071.
39. Haldar P, Brightling CE, Hargadon B, Gupta S, Monteiro W, Sousa A et al. Mepolizumab and exacerbations of refractory eosinophilic asthma. *N Engl J Med* 2009;**360**:973–984.
40. Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000;**356**:2144–2148.
41. Nair P, Pizzichini MM, Kjarsgaard M, Inman MD, Efthimiadis A, Pizzichini E et al. Mepolizumab for prednisone-dependent asthma with sputum eosinophilia. *N Engl J Med* 2009;**360**:985–993.
42. Karras JG, Crosby JR, Guha M, Tung D, Miller DA, Gaarde WA et al. Anti-inflammatory activity of inhaled IL-4 receptor- α antisense oligonucleotide in mice. *Am J Respir Cell Mol Biol* 2007;**36**:276–285.
43. Hamelmann E, Tateda K, Oshiba A, Gelfand EW. Role of IgE in the development of allergic airway inflammation and airway hyperresponsiveness—a murine model. *Allergy* 1999;**54**:297–305.
44. Busse W, Corren J, Lanier BQ, McAlary M, Fowler-Taylor A, Cioppa GD et al. Omalizumab, anti-IgE recombinant humanized monoclonal antibody, for the treatment of severe allergic asthma. *J Allergy Clin Immunol* 2001;**108**: 184–190.
45. Anderson BJ, Holford NHG. Mechanism-based concepts of size and maturity in pharmacokinetics. *Ann Rev Pharmacol Toxicol* 2008;**48**:303–332.