Use of a Canine Model of Atopic Dermatitis to Investigate the Efficacy of a CCR4 Antagonist in Allergen-Induced Skin Inflammation in a Randomized Study

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Atopic dermatitis (AD) is an inflammatory skin disease characterized by infiltration of skin homing lymphocytes into the dermis. Most of these lymphocytes express the chemokine receptor CCR4, and the frequency of blood CCR4⁺ lymphocytes correlates with AD disease severity. Canine AD is a pruritic inflammatory condition that shows many features of the human disease, including CCR4 overexpression. Therefore, we tested a potent selective CCR4 antagonist in an allergen challenge model of canine AD, both clinically and histologically, to investigate whether this chemokine pathway plays a role in the inflammatory response. Using a four-period randomized cross-over study design, 14 beagles were challenged with allergen and clinically monitored. Biopsy samples were taken before and after allergen challenge. A clear reduction of clinical scores was observed with oral prednisolone (P < 0.0001) but not for the CCR4 inhibitor. A subset of the dogs (5/13) showed partial inhibition (30–49%) of the clinical signs with CCR4 inhibitor treatment, and this finding was supported by the results of histopathologic analysis of skin biopsy samples. This partial response is consistent with redundancy in chemokine pathways and highlights the need for therapies blocking multiple pathways. This study shows the utility of this canine model of AD for testing new therapeutic agents.

INTRODUCTION

In man, atopic dermatitis (AD) is a common, chronic inflammatory skin disease that generally runs a relapsing, remitting course. The therapeutic objectives in AD are twofold: to reduce active signs and symptoms and to prevent or reduce the frequency of flares or disease recurrence.

Because of the complexity of AD, the identification of new therapies relies initially on the use of animal models. Dogs spontaneously develop a skin condition that is clinically and immunologically almost identical to human AD.

A canine model of AD has been identified. In this validated model, high IgE atopic beagles spontaneously develop clinical signs of AD and are easily sensitized after epicutaneous application of allergens such as house dust mites (HDM) (Marsella et al., 2006). These dogs have been described to have immunologic, clinical, and ultrastructural changes similar to those of spontaneously occurring AD in dogs and human patients (Marsella and Girolomoni, 2009). After sensitization, flare-up of clinical signs of AD can be triggered by environmental challenge with HDM. This model allows testing of new treatments as the allergen exposure can be closely titrated and the development of lesions followed over time. Severity of clinical signs is assessed using a validated clinical scoring system similar to the SCORing of Atopic Dermatitis (SCORAD) (Oranje et al., 2007) used in clinical assessment in man. This scoring system, the Canine Atopic Dermatitis Extent and Severity Index (CADESI), has been validated in previous studies (Olivery et al., 2007).

The histopathology of AD is characterized by infiltration of immune cells such as lymphocytes and eosinophils into the dermis. The majority of the skin homing lymphocytes that express cutaneous lymphocyte-associated antigen (CLA) also express the chemokine receptor CCR4 (Campbell et al., 1999). The frequency of peripheral blood CCR4⁺ lymphocytes reflects the severity of disease in AD patients (Nakatani et al., 2001; Wakugawa et al., 2001), and CCR4 expression is...
increased in chronic skin lesions in AD skin compared to normal or psoriatic skin (Nakatani et al., 2001). A significant body of data shows that the plasma concentrations of the CCR4 ligands TARC (thymus and activation regulated chemokine) and MDC (macrophage-derived chemokine) correlate with disease severity in AD (Hijnen et al., 2004; Horikawa et al., 2002; Kakinuma et al., 2002). Although there is significant evidence for the role of CCR4 in human AD, the results of in vivo studies in preclinical species have been inconclusive (Islam et al., 2011; Nakagami et al., 2009, 2010).

The aim of this study was to test the hypothesis that CCR4 antagonists would be an efficacious therapy for AD using a relevant in vivo model as a proof of concept study. The canine allergen challenge model of AD was selected for the study because of the similarities with human disease, evidence that CCR4 and TARC are up-regulated in lesional skin of dogs with AD (Maeda et al., 2002), and the ability to measure clinically relevant outcomes through the CADESI score.

RESULTS
AZ445 is a potent, selective CCR4 antagonist with suitable in vivo pharmacokinetic and safety profile in the dog
AZ445 was demonstrated to be a potent inhibitor of human and dog CCR4 and was functionally active in an assay of human Th2 cell chemotaxis, albeit with reduced potency due to high plasma protein binding (Table 1). Selectivity for CCR4 was confirmed by testing against a panel of >180 targets, including the chemokine receptor CCR5. All assays showed a >100-fold selectivity compared to the potency at CCR4 (data not shown).

Confidence in both dosing and sampling within dogs used in the study was obtained by comparing the exposure at 0.48 and 1.6 mg/kg (1.04 and 3.5 μmol/kg, respectively) with predicted profiles modeled from a preliminary pharmacokinetic study in AstraZeneca animals using a two-compartment pharmacokinetic model incorporating rapid absorption (see Supplementary Figure S1 online).

This initial analysis indicated that the exposures were relatively similar to those predicted, although the terminal half-life appeared somewhat shorter in the dogs used in the pharmacodynamic model. Nevertheless, there was evidence of accumulation over repeated dosing, and exposure from the higher dose resulted in higher plasma concentrations. Trough concentrations at the two doses provided confidence in achieving plasma concentrations equivalent to ~3× (range 2.5–6.8×) and 7× (range 3.0–10.1×) IC50 for CCR4 during the course of treatment. The 3× IC50 has been shown to be an effective plasma exposure for a range of established G-protein coupled receptor targets with marketed drugs (McGinnity et al., 2007). The higher dose was chosen to provide a plasma exposure equivalent to a significantly higher multiple of CCR4 IC50 as indicated for several chemokine targets (Schall and Proudfoot, 2011) while also taking the amount of compound required into consideration.

In order to confirm the tolerability of the CCR4 antagonist in dogs before the start of the study, three dogs were dosed with 7 mg/kg AZ445 once daily for 21 days. The compound was well tolerated, with no significant in-life or histopathological findings and only minor changes in hematology and plasma chemistry.

Prednisolone, but not CCR4 antagonist, caused a significant reduction in CADESI score
The results of CADESI scoring in this study were consistent with the previously reported pattern of reactions, with progressive increase of the scores during the course of allergen challenge followed by a slow decrease after allergen exposure was stopped (Marsella et al., 2006). Representative images of the clinical presentation of the dogs in the different treatment arms are shown in Supplementary Figure S2 (online).

For each treatment period of the cross-over study, the total CADESI score from the five subscales (see Supplementary Table S1 online) was measured for each individual animal at various time points up to 9 days (Figure 1a). The total area under the curve (AUC) was calculated, and the “baseline” AUC, obtained using the CADESI score at baseline, was subtracted to give an adjusted AUC CADESI. The baseline adjusted AUC CADESI was used in the formal cross-over statistical analysis. The adjusted AUC CADESI scores for each animal are shown in Figure 1b. The component scores for papules and diffuse erythema contributed most to the total CADESI score. The pattern for these individual components was broadly the same as the total CADESI score.

A statistically significant reduction in baseline adjusted AUC CADESI score was found in prednisolone-treated dogs compared to vehicle (P < 0.001), but no statistically significant reduction in AUC was found with either the low (0.48 mg/kg) or high (1.6 mg/kg) dose of AZ445 compared to vehicle (P = 0.214 and P = 0.293, respectively). The group mean AUC CADESI score and standard error are shown in Figure 1c. No statistically significant effect of carryover between the treatment arms was found (P = 0.814).

Despite an overall nonstatistically significant reduction in adjusted AUC in the low- and high-dose AZ445 groups compared to vehicle, on an individual animal level, five dogs showed a marked reduction in adjusted AUC compared to the vehicle, when considered relative to the vehicle to prednisolone adjusted AUC window. Representative data for these animals showing a partial response are shown in Figure 1d.

### Table 1. In vitro properties of CCR4 inhibitor AZ445

<table>
<thead>
<tr>
<th>Human CCR4 binding potency (pIC50)</th>
<th>Dog CCR4 binding potency (pIC50)</th>
<th>Human MDC-stimulated chemotaxis (pIC50)</th>
<th>Human plasma protein binding (% free)</th>
</tr>
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<tbody>
<tr>
<td>8.3 ± 0.1 (n = 7)</td>
<td>8.8 ± 0.3 (n = 4)</td>
<td>6.8 ± 0.6 (n = 3)</td>
<td>0.62 ± 0.33 (n = 3)</td>
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Data shown are mean ± SD.

Abbreviation: MDC, macrophage-derived chemokine.

1Potency in assay containing 0.3% human serum albumin. When corrected for plasma protein binding, pIC50 = 8.2, which is equivalent to the potency in the binding assay.
CCl4 antagonist and prednisolone reduced epithelial thickening, dermatitis, and CCR4+ cell infiltration after allergen challenge

The histopathologic changes observed in the model primarily involved the epidermis and superficial to mid-dermis. Skin specimens from the vehicle-treated group (Figure 2a) were generally characterized by epidermal hyperplasia, along with frequent intracellular edema, variable hyperkeratosis, and occasional excoriation/serocellular crusting. Dermal infiltrates were predominantly mononuclear in composition (macrophages, lymphocytes, and plasma cells), with scattered eosinophils and occasional neutrophils. Dermal blood vessel plexuses often had prominent endothelial cells (hypertrophy), indicative of local inflammation, with a surrounding perivascular cuff of mixed inflammatory cells. The extent of dermatitis was generally associated with a proportional increase in CCR4+ mononuclear cells (Figure 3a). With AZ445 treatment, epithelial thickening was reduced, as were dermal inflammatory cell infiltrates and associated CCR4 positivity (Figures 2b and c, and 3b and c). Degenerate changes (e.g., excoriation/erosion) within the epidermis were fewer, and inflammatory cells became more sparsely distributed. With prednisolone treatment (Figures 2d and 3d), structural epidermal and dermal changes were notably reduced in terms of severity, often to within the range observed in the baseline control sample (see Supplementary Figure S3 online).

The individual animal histopathology and CCR4 immunohistochemistry (IHC) scores are shown in the heat map in Figure 4 based on the scoring system described in Supplementary Table S2 (online). The Fisher exact test (FET) was performed on the summary counts of the scores in Figure 4 for each endpoint.

For epithelial hyperplasia, the FET on the summary counts with all four groups gave \( P = 0.002 \), and with prednisolone removed \( P = 0.130 \). For dermatitis, the FET with all groups gave \( P = 0.005 \), and with prednisolone removed \( P = 0.140 \). Finally, for CCR4, the FET using all dose groups gave \( P = 0.015 \), and with prednisolone removed \( P = 0.464 \). Therefore, there is a significant association between dose group and histopathologic response when prednisolone is included, but no association when prednisolone is excluded.

Although FET analysis did not demonstrate statistical significance, the histopathology and IHC data showed a trend toward reduction of the allergen-induced skin inflammation with CCR4 inhibition, albeit to a lesser extent than with prednisolone.

DISCUSSION

This study failed to demonstrate a statistically significant improvement in CADESI score in this model when a CCR4 inhibitor was used at either of the doses selected. In the histopathologic analysis, prednisolone was able to reduce or completely eliminate the histopathologic effects of epithelial hyperplasia and dermatitis. Low- and high-dose AZ445 tended to rank somewhere between vehicle and prednisolone, suggesting a potential effect on the histopathology that was not completely mirrored by CADESI scoring. Both prednisolone and the AZ445 doses reduced the number of CCR4+ cells in the skin biopsy samples, suggesting that the CCR4 inhibitor was achieving a pharmacologic effect. This finding was supported by the limited pharmacokinetic data, which confirmed predicted exposure at approximately 3 × and 7 × IC50 for the compound. This fold increase above the IC50 is consistent with efficacy for a range of licensed G-protein coupled receptor targets; hence, lack of exposure likely is not the reason for lack of efficacy.

In both dogs and man, glucocorticoids are routinely used in clinical practice to control AD flares. However, because glucocorticoids have a broad-spectrum mechanism of action, expecting a targeted treatment to have the same effect would be unrealistic and does not decrease the value of such a targeted treatment option. Targeting of a specific chemokine pathway also may not result in complete inhibition of the inflammatory response because of the potential for...
Figure 3. CCR4 IHC/hematoxylin counterstained samples harvested and processed from the lesion periphery (animal 5). (a) Vehicle. Note both the diffusely distributed and the focally aggregated (indicated with stars) CCR4⁺ inflammatory cell infiltrates within the superficial to deep dermis. (b, c) Low- and high-dose AZ445, respectively. Note both the focally aggregated (stars) and the individual (thin arrows) CCR4⁺ inflammatory cell infiltrates within the superficial dermis. Although the dermal “hotspot” is more extensive in b, assessment of the entire specimen from b and c was such that they were both graded as “slight” (see Supplementary Table S1). (d) Prednisolone. Note the rare and individual CCR4⁺ inflammatory cells, predominantly located within the superficial dermis. The quantity and distribution of these cells were within the ranges seen in the baseline sample. Thin arrow denotes CCR4⁺ cells. IHC, immunohistochemistry. Bar = 0.5 mm.

Figure 2. Hematoxylin and eosin–stained skin samples harvested and processed from the lesion periphery (animal 5). (a) Vehicle. Note the extensive and mixed dermal inflammatory cell infiltrate (chronic-active dermatitis; indicated with stars), which forms a predominantly perivascular distribution. Overlying epithelium is thickened and thrown up into folds with diffuse hyperplasia (proliferation) and focal areas of excoriation (thin arrow). Several keratinocytes also display intracellular edema (thick arrow). (b) Low-dose AZ445. Note the reduction in dermal inflammatory cell infiltration (star) and presence of epidermal hyperplasia with pigmentation (thin arrow). (c) High-dose AZ445. In this sample, there is a similar severity of dermal inflammatory cell infiltration (star) and epithelial hyperplasia (thin arrow) compared to b. Superficial dermal blood vessel plexuses show reactive (hypertrophic) endothelium (adjacent to the area of inflammation). (d) Prednisolone. Note the scattered dermal inflammatory cell infiltrates (star) and epithelial morphology (thin arrow), which both are within the range seen in the baseline sample. Bar = 0.5 mm.
Although CCR4 is expressed on the majority of skin homing T cells, there is also evidence for expression of CCR8 and CCR10 on subsets of CLA⁺ T cells (Soler et al., 2003, 2006) as well as CRTh2 on CLA⁺ T cells and eosinophils in allergic skin disease (Iwasaki et al., 2002; Yahara et al., 2010).

Although the majority of the dogs treated with AZ445 did not show a reduction in CADESI score, 5 of the dogs (38%) showed a partial response at both doses of the CCR4 inhibitor, with an approximately 40% reduction of the CADESI score when assessed relative to the vehicle-to-prednisolone adjusted AUC. In both dogs and man, AD most likely is a clinical syndrome in which different pathways and mechanisms of disease may lead to the same clinical presentation but varying responses to the same treatment by different individuals. Thus, when more targeted treatment options are used, some individuals for which that pathway is important may show a strong clinical response, some may show a partial response (multiple pathways are activated), and some may show no response (different mechanism activated in that patient). In these partially responding dogs, no obvious consistent feature in either clinical or histologic phenotype would have predicted a response. In the future, the ideal approach would be personalized medicine in which drugs are selected based on biomarkers expressed by the patient.

One benefit of the model used in this study is that the clinical outcomes in terms of CADESI score can be linked to some of the clinical outcomes described by scoring systems such as SCORAD and EASI because of an overlap in a number of domains. In recent studies such as those using the dual IL-4/IL-13 receptor antibody dupilumab, the EASI50 reported in a clinical trial (clinical trial reference NCT01385657) was 85.5% compared to 35.2% for placebo (Beck et al., 2014). Therefore, in theory for this model the percentage of subjects responding at a predefined level would be another way to present the data. However, EASI50 scores normally are calculated as change from the beginning to the end of treatment of a chronic disease. The canine model described in this study is generally a self-limiting condition as demonstrated by the vehicle response, usually a peak response within a few days of HDM challenge and then gradual resolution when the allergen is removed. Because determining an appropriate time frame to calculate the CADESI50 score is more of a challenge, the approach taken in this study was to quantify the AUC of the CADESI score over a specific duration; therefore, this method is not directly comparable to any commonly used clinical approaches.

Another limitation of this study in terms of translation of dog model data to AD in dogs or humans is that these dogs live in a controlled environment that is different from the real world of allergen exposure (Marsella et al., 2006). This explains why their disease is milder between allergen challenges. Once these dogs are adopted out of the laboratory environment and are exposed to a more consistent allergenic load, they exhibit symptoms of naturally occurring chronic relapsing pruritic dermatitis with periods of exacerbation and remission. This model is closest to that of naturally occurring disease and has been shown to respond to calcineurin inhibitors (White et al., 2015), as reported for dogs in a clinical situation.

Not uncommonly, in a clinical situation a multimodal approach is used to control clinical signs. Future studies could consider the use of glucocorticoids or other broad-spectrum treatments to induce remission and a more targeted therapy as a “steroid-sparing agent.”

Although the holy grail is the identification of new treatments that can provide an alternative to glucocorticoid use, unless a broad spectrum of action is used (at the cost of unwanted adverse effects), the success rate may not be as high as with glucocorticoids. With this consideration in mind, the results of the present study are encouraging, and this approach may be a helpful strategy for individuals in whom CCR4 is a clinically relevant mechanism of disease.

Further studies to investigate combination approaches are warranted, as are studies to evaluate and define, based on the genotypic or phenotypic features of the disease, the patient population that would respond better to treatment.

MATERIALS AND METHODS
All procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Florida.

Reagents
AZ445 was synthesized in the Medicinal Chemistry Laboratories of AstraZeneca R&D (Charnwood, Loughborough, UK). AZ445 is a...
Characterization of the pharmacologic, physicochemical, and pharmacokinetic properties of AZ445

Potency of AZ445 binding to membranes of CHO cells expressing human or dog CCR4 was determined using fluorometric micro-volume assay technology as described previously (Andrews et al., 2008). For chemotaxis assays, CD45RA⁺ T cells purified from human peripheral blood mononuclear cells by negative selection were polarized to a Th2 phenotype by incubation with human IL-4 and human anti-IL-12 antibody (R&D Systems, Abingdon, UK). Chemotaxis of cells to the CCR4 agonist MDC was performed for 1 hour at 37 °C in the presence of 0.3% (v/v) DMSO or compounds. The number of migrated cells was determined by measurement of lactate dehydrogenase activity after cell lysis. Concentration-dependent inhibition of chemotaxis by compounds was expressed as pIC₅₀ (negative log of IC₅₀).

Experimental design

The study conformed to the Consolidated Standards of Reporting Trials guidelines. The study design was a four-way cross-over, initially with 14 dogs (8 females and 6 males). The four treatment groups were vehicle, low dose of test compound, high dose of test compound, and prednisolone. Dogs were randomized to treatment sequence using a multiple Williams Latin square design. The design of the study is shown in Figure 5.

Animals and housing

Atopic beagle dogs were housed in pairs in a research facility of University of Florida and regularly exercised and socialized as part of an enrichment program. They were separated during the course of studies to prevent cross-contamination and allow more accurate evaluation. Because the runs were cleaned daily at high temperature and no stuffed toys that could trap HDM were allowed in their environment, no exposure to HDM occurred except as part of the allergen challenges of the experiments. All dogs were fed the same diet (Hills Science Diet Maintenance) and had free-choice water access. For dosing, vehicle was prepared before each arm using Methocel E4M premium, at 0.5% w/v in deionized purified water. Two doses of AZ445 were given at each arm to allocated dogs at a concentration of 0.48 and 1.6 mg/ml in deionized purified water. Dogs were randomized to treatment sequence using a multiple Williams Latin square design. The design of the study is shown in Figure 5.

Clinical evaluation

CADESI scores were taken on day 1 (baseline score, before compound administration), day 2, twice on the 3 days of allergen challenge (days 3–5, before allergen challenge and 6 hours after), then once daily for the remaining days of compound administration (9 days in total). Clinical evaluation was performed by an assessor who was blinded to treatment allocation using a modified version of the validated Canine Atopic Dermatitis Extent and Severity Index Score-03 (CADESI-03) (Olivry et al., 2007). The two modifications from CADESI-03 were inclusion of papules as a clinical sign and scoring for each sign from 0 to 3 rather than from 0 to 5 (Marsella and Sandomichelakis, 2010).

The dog’s body was divided into sections, each of which was scored based on the clinical signs evaluated (diffuse erythema, erythematous macules, papules, excoriations, and alopecia). The total score was calculated by adding the score of various body regions and clinical signs (see Supplementary Table S1).

Statistical analysis

The total CADESI score to the end of the study was used to construct a baseline adjusted AUC response. The adjusted AUC was compared to the vehicle by a four-way cross-over analysis using SAS software, version 9 (SAS Institute Inc, Cary, NC). Comparisons to vehicle were one-sided to detect a reduction in AUC in the test compounds. The significance level was set at 5%. The analysis included a test for carryover effect.

The histopathologic data were analyzed by FET using SAS, version 9.

Pharmacokinetic analysis

Serial blood samples were taken from dogs on days 1 and 9 of the study at 0, 0.5, 1, 3, 7, and 24 hours after dosing. A simplified (sparse) sampling protocol was adopted because the principal aim of the bioanalysis was to provide confidence in dosing across the different treatment groups together with an indication of interanimal variability in exposure. Plasma was obtained by centrifugation and analyzed by HPLC-MS-MS as described previously (Paine et al., 2011). Derived parameters were estimated from the concentration-time profile (Cmax, Tmax) and/or by noncompartmental analysis (AUCall) using WinNonLin (Pharsight Corporation, Cary, NC).

Histopathologic and immunohistochemical study of CCR4 expression in skin biopsies

At baseline before the study and on day 5 for each arm, 8-mm dog skin biopsy samples were collected, fixed in 10% neutral buffered formalin for 48 hours, and processed into paraffin wax. Sections 4-μm thick were used for standard hematoxylin and eosin staining and immunohistochemistry.

Before immunohistochemical staining, antigen retrieval was carried out in 0.01 M citrate buffer (pH 6.0) at 110 °C for 2 minutes. Slides were transferred to a Lab Vision immunostainer (Thermo Fisher Scientific, Loughborough, UK), and all steps were carried out at room temperature. Nonspecific Ig-binding sites were blocked for 20 minutes using a background blocker with casein (A. Menarini Diagnostics, Berkshire, UK). CCR4 was specifically detected using a monoclonal mouse anti-human CCR4 antibody (1:250, 551121; BD Pharmingen, UK) for 60 minutes, followed by biotinylated horse anti-mouse (1:300, BA-2001; Vector, Peterborough, UK) secondary reagent for 20 minutes and detection with a TSA amplification-HRP system (NEL700; Perkin Elmer, Seer Green, UK). Positive staining was visualized with 3,3’-diaminobenzidine (A. Menarini Diagnostics) and counterstained with Carazzi hematoxylin. Appropriate negative controls were used. The grading scheme for the histopathology and IHC is shown in Supplementary Table S2. The histopathologist who performed the assessment was blinded to treatment allocation.

CONFLICT OF INTEREST

Clare Murray, Matt Devalaraja, Mike Dymond, Mallinder Fagura, Adam Hargreaves, Alison Holt, Jaimini Reens, Rob Riley, Ian Peers, and Sally Price are current or former employees of AstraZeneca.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2015.11.001.

REFERENCES


