

Lack of preventing effect of systemically and topically administered histamine H₁ or H₄ receptor antagonists in a dog model of acute atopic dermatitis

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Abstract: As there is evidence for an anti-inflammatory efficacy of histamine H₄ receptor (H4R) selective antagonists, we aimed at testing the efficacy of the H4R antagonists JNJ7777120 and JNJ28307474 in comparison with histamine H₁ receptor (H1R) antagonists hydroxyzine and cetirizine for skin lesion prevention in a canine model of acute atopic dermatitis. Six atopic Maltese-beagle dogs experimentally sensitized to *Dermatophagoides farinae* (Df) house dust mites were selected for this study. Twenty-four hours after challenge by epicutaneous application of Df, erythematous skin lesions were scored. In this blinded, placebo and active controlled study, topical JNJ7777120 or JNJ28307474 was applied as a 1% solution before allergen challenge. The latter was also given orally at 15 mg/kg before and after allergen challenge. A 0.015% triamcinolone acetonide solution was used as a positive control. The H1R antagonists hydroxyzine and cetirizine were administered orally before challenge in a second experiment.

Twenty-four hours after challenge, placebo-treated animals had a median lesional score of 2. Treatment with topical and oral JNJ28307474 resulted in a median score of 2.5. After topical administration of JNJ7777120, the median lesional score was 2. Hydroxyzine and cetirizine did also not reduce the median score of the placebo treatment. Triamcinolone acetonide prevented all dogs from having any lesions. Determination of histamine in lesions revealed that only during the initiation increased concentrations of histamine were detected. In conclusion, the preventive administration of H1R or H4R antagonists has no impact on the development of acute skin lesions in this experimental canine atopic dermatitis model.

Key words: canine atopic dermatitis – histamine H4 receptor – microdialysis – JNJ28307474 – JNJ7777120

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Introduction

Allergic skin diseases such as atopic dermatitis (AD) are characterized by local inflammation and pruritus. There are several observations that suggest that, in dogs as well as in humans, the prevalence of AD has risen in the past several decades; it is now estimated to be around 10% in dogs (1), and it is reported to affect 10–20% of children and 1–3% of adults in industrial countries (2). Although classical treatment modalities like glucocorticoids or calcineurin inhibitors show good efficacy, their use is limited by their side effects, which get more pronounced because of the need of long-term treatment (3–5). Thus, there is still a need for antiallergic drugs with a better risk–benefit ratio.

Since the cloning of the histamine H₄ receptor (H4R) in 2000, there has been ample evidence for an important role of this new histamine receptor in the regulation of immune responses and in the modulation of allergic disorders (6). The distinct expression profile of H4R, which predominates on hematopoietic cells, makes this receptor ideally suited as a new target for atopic diseases (7).

Besides glucocorticoids, commonly used drugs for allergic diseases are antihistamines, which are histamine H₁ receptor (H1R) antagonists (blockers), but they are minimally effective in many

clinical conditions (e.g. AD) in dogs as well as in humans (8–10). The lack of efficacy of histamine H₁ antihistamines for the treatment of allergic skin lesions might partly be because of the lack of blocking of the H4R-mediated histamine actions. This theory is supported by the observation that the first series of H4R selective antagonists provided evidence for anti-inflammatory and antipruritic *in vivo* efficacy (11,12).

The objectives of this study were to test the effectiveness of two H4R antagonists for acute skin lesion prevention in a canine model of AD and to compare the efficacy to that of two H1R antagonists known to reduce histamine- or allergen-induced wheal and flare reactions in dogs (13,14). To gain further information about the role of histamine in the development of acute AD skin lesions, the amount of secreted histamine at sites challenged with *Dermatophagoides farinae* (Df) allergen was measured by dermal microdialysis technique directly after application as well as 6 and 24 h after challenge.

Material and methods

All phases of the experimental design of the study were approved beforehand by North Carolina State University Institutional Animal Care and Use Committee (IACUC).

Study subjects

Six atopic Maltese-beagle crossbred dogs (MBA) were enrolled in this study. This inbred line of dogs was selected because it is known to exhibit spontaneous hypersensitivity to food allergens and to develop AD-like signs upon challenge with such allergens (15). Moreover, our previous studies confirmed the easy sensitization of these dogs to Df allergens and the development of AD-like skin lesions following epicutaneous mite allergen challenges (16). These dogs do not usually develop skin lesions, apart from occasional bacterial folliculitis, if not provoked with an allergen to which they are hypersensitive.

In all, there were three males (two intact) and three females (one intact). The average age was 9.7 years (range 7–13 years).

Experimental design

Two highly selective H4R antagonists were chosen for the described study. JNJ777120 has an affinity of 50 nM at the canine H4R (17). JNJ28307474 was chosen, owing to its good tolerance, similar high affinity to the canine H4R (18) and favourable half-life in dogs ($t_{1/2}$ in dogs after oral administration is approximately 6 h, R. Thurmond, unpublished data). For comparison, the H1R antagonists cetirizine and hydroxyzine were selected as pharmacodynamic data already exist in dogs for both substances (13,14).

Dermal penetration of JNJ777120 and JNJ28307474 according to OECD guideline 428

For topical administration, JNJ777120 was synthesized by KS and HS as described previously (19) and formulated according to an approved protocol taken from the German Drug Codex (DAC/NRF) as a 1% (m/m) solution in isopropyl myristate (IPM) and 2-propanol. JNJ28307474 was provided by R. Thurmond (Johnson & Johnson Pharmaceutical Research & Development, L.L.C., La Jolla, CA, USA) and formulated as a 1% (m/m) solution as described for JNJ777120. Blank samples contain IPM and the solvents.

Skin samples were taken from dead animals from the lateral abdominal skin (six Beagles, 5 months old, humanely sacrificed at the University of Veterinary Medicine Hannover for reasons not related to this study). After excision, each skin sample was shaved carefully and then stored at -20°C for 1 month before use. After defrosting at room temperature, each skin sample was dermatomed to a thickness of $500 \pm 100 \mu\text{m}$ using a dermatome (Zimmer, Freiburg, Germany).

To compare diffusion results obtained by frozen skin from young dogs (according to OECD guideline 428) with dog skin obtained directly after euthanasia of older dogs, skin samples were also taken from two 12- and 15-year old crossbreeds and one 7-year-old boarhound and treated with JNJ777120 as described below.

The following buffers (analytical grade; Merck, Darmstadt, Germany) were used for the experiments: Phosphate-buffered saline (pH 7.49), McIlvaine citrate buffer (pH 2.2).

The diffusion experiments were performed in Franz cells with a diffusion area of 1.77 cm^2 (Permeagor, Bethlehem, PA, USA). Skin was equilibrated in phosphate-buffered saline for 30 min prior to the experiment. Skin samples were mounted epidermal side-up into the diffusion cell, and $200 \mu\text{l}$ of the test substance solutions (1% m/m) was applied. Twelve millilitres of phosphate-buffered saline was used as acceptor medium. The temperature of the solution in the acceptor chamber was kept constant at 32°C by a

circulating water bath that was stirred continuously. Samples ($400 \mu\text{l}$) from the acceptor chamber were taken at predefined times (up to 28 h) and were replaced with $400 \mu\text{l}$ phosphate-buffered saline.

The test compounds were quantified with the following HPLC system: Pump 126, Solvent Module and Detector (168), Beckmann, München, Germany; Column heater: 40°C , Spark Holland, Emmen, Netherlands; HPLC-Column: LiChrospher 100, CN ($5 \mu\text{m}$) and guard column: LiChrospher 100 CN ($5 \mu\text{m}$), Merck; Software: 24 Karat 5.0, Beckman.

Detection was performed with 70% methanol (Merck) and 30% McIlvaine citrate buffer at 297 nm. Aliquots of $100 \mu\text{l}$ were injected and analysed at a flow rate of 1.5 ml/min. The limits of quantification were 400 ng/ml (JNJ777120) and 200 ng/ml (JNJ28307474).

Effects of H1R and H4R antagonists on atopic skin lesions provoked by challenge with Df antigen

This study was designed as a randomized blinded, placebo-controlled, cross-over experiment with a triamcinolone aqueous solution serving as a positive control. Between each treatment procedure, a minimum of 14 days of washout were taken, which led to a total resolution of any inflammatory signs at the site of previous allergen application.

Sensitization protocol

In dogs enrolled in this study, hair was clipped 1 day before the stratum corneum (SC) was removed from the right axilla and groin with 10 consecutive tape strips performed using a one-inch wide clear adhesive tape (Scotch packaging tape, 3M). Each successive strip was performed with a new tape area to ensure appropriate stickiness and SC removal.

Following tape stripping, $500 \mu\text{g}$ of lyophilized Df whole mite powder (Greer laboratories, Lenoir, NC, USA) was suspended in mineral oil. Twenty microlitres of this suspension was rubbed onto the previously tape-stripped right axilla once weekly for eight applications, this sensitization lasting 2 months. After monthly booster by epicutaneous Df application, all dogs enrolled in this study had elevated Df-specific IgE, positive Df intradermal test reactivity and positive skin lesions at the site of previous Df application.

Administration of the H4R antagonists and triamcinolone spray

JNJ777120 or JNJ28307474 (1%, $500 \mu\text{l}$) was administered topically as a solution (isopropanol/IPM) at a concentration of 1% 24 h before and 1 h before topical challenge with house dust mite antigen onto the axillary region. JNJ28307474 was additionally administered orally (15 mg/kg in 5 ml 20% 2-hydroxypropyl-cyclodextrin) 30 min before and 4 h after topical challenge. This time period and dose were chosen because of the good tolerance and favourable half-life of JNJ28307474 in dogs (R. Thurmond, unpublished data). Additionally, this dose regimen previously led to anti-inflammatory effects in a murine model of allergic dermatitis (18). As a result of an unfavourable pharmacokinetics of JNJ777120 in dogs (11), JNJ777120 was not tested systemically. A 0.015% triamcinolone acetonide solution (Genesis[®], Virbac, Fort Worth, TX, USA) was used as a positive control applied 24 and 1 h before allergen challenge.

Administration of the H1R antagonists

In a separate experiment performed after an adequate washout period with the same six Maltese-beagles, the H1R antagonists

cetirizine and hydroxyzine and corresponding placebo were tested similarly to the H₄R antagonists. Cetirizine (0.5 mg/kg) was administered 24 and 2 h before challenge according to a previous study (13), and hydroxyzine (2 mg/kg) was administered orally 24, 12 and 2 h before allergen challenge on the basis of Bizikova et al. (2008) (14).

Assessment of inflammatory skin lesions

Lesions were evaluated 24 h after topical challenge. Erythematous macules, oedema, papules/pustules and excoriations were scored as 0 (absent), 1 (faint, mild) or 2 (strong, severe). The grades for each lesion were added to yield a lesional score. The maximal lesional score achievable was eight points. Evaluation was performed by one of the authors (TO) who was not aware of the intervention received by the subjects. Using this method of topical application of a small amount of Df antigen onto the axillary region, a reproducible itch response is normally not elicited. Thus, no information is obtainable about a possible antipruritic quality of the tested substances.

Stability testing of the topically administered compounds

After the clinical study was performed, reference samples of the solutions were concentrated to dryness under reduced pressure giving whitish semi-solid residues. These probes were subsequently analysed by ESI MS and ¹H NMR (one- and two-dimensional) to evaluate the stability of the active ingredients and IPM. The ESI MS spectra showed the expected mass peak of the monoprotonated free bases (M+H⁺) without peaks of decomposition products. IPM or corresponding fragments were not detected. The ¹H NMR spectra contained all relevant peaks of the JNJ compounds and IPM, respectively. Decomposition products were not detected. JNJ7777120 and IPM were present at a molar ratio of 1:1.5, whereas the probe of the JNJ28307474 solution contained the H₄R ligand, IPM and 2-propanol in an equimolar ratio. In conclusion, no signs of decomposition were detected after the study in the H₄R antagonist containing solutions.

Determination of histamine and Derf 1 antigen in atopic lesions provoked by challenge with Df antigen

Microdialysis experiments were performed as previously described (20). In brief, sterile membranes (outer diameter 216 µm, 2-kDa molecular weight cut-off) were inserted intradermally after sedation. Phosphate-buffered saline was used as perfusion fluid. For the determination of Derf 1 antigen, a 3000-kDa molecular weight cut-off microdialysis fibre with an outer diameter of 0.4 mm was used. All microdialysis membranes were from Laboratory of Experimental Physiology and Inflammation, Denmark. Phosphate-buffered saline was used as perfusion fluid in all experiments. The microdialysis pump (TSE, Bad Homburg, Germany) was set with a flux of 5 µl/min. In a pilot experiment, a dog received intradermally anticanine IgE antiserum (8 µg in 100 µl), which increased baseline dialysate histamine from 10 nM to a peak value of approximately 350 nM. This is within the range of histamine that was measured after intradermal injection of *Ascaris* antigen to sensitized beagle dogs in a microdialysis study (21); as a result, it was decided that an intradermal injection of anti-IgE antiserum would serve as a positive control at the end of each experiment.

Three atopic Maltese-beagle crossbred dogs, which had also been enrolled in the main study described above, were sedated with dexmedetomidine (10 µg/kg i.m.). After insertion of the microdialysis fibres in the shaved lateral thorax, an equilibration

time of 15 min was taken to avoid artificial increase in histamine because of trauma. The concentration of histamine directly after insertion ranged from 5 to 22 nM histamine.

Samples were taken for 2–3 h (depending on the duration of sedation) after topical administration of Df allergen. A second and third measurement was performed with two beagles after sedation (dexmedetomidine, 10 µg/kg i.v.) between 6 and 7 h and between 24 and 25 h after topical administration of the Df allergen. To determine a possible histamine release induced by tape stripping, the effect of the latter without application of Df antigen was studied in a separate microdialysis experiment. The maximum detected histamine concentration after tape stripping was 14 nM.

Histamine and Derf 1 antigen were measured in the microdialysis samples by ELISA (Demeditec, Kiel, Germany and Indoor Biotechnologies, Charlottesville, VA, USA, respectively). The sample size for the histamine assay was 25 µl, and the limit of detection (for plasma) was approximately 1 nM.

Statistical analysis

The lesional score of the treatment groups was compared to placebo by a Kruskal–Wallis test, followed by a *post hoc* test (Dunn's test).

Results

Dermal penetration of topically administered JNJ7777120 and JNJ28307474 through dog skin

To estimate the possible dermal penetration of the two selective H₄R antagonists, Franz diffusion cell experiments with dog skin were performed. Both substances penetrated dog skin during 28 h of diffusion experiments. However, the magnitude of permeated substance was several magnitudes higher in dog skin treated with JNJ7777120 compared to JNJ28307474 (Figure S1). Whereas 33.5% of the administered JNJ7777120 could be found in the acceptor medium 28 h after application, only 0.75% of the used JNJ28307474 was recovered.

The penetration of JNJ7777120 through freshly prepared dog skin of older dogs was lower than that found through dogs skin of young beagles. Only 10.2 (±2.1) % of the topically administered JNJ7777120 was found in the acceptor medium 24 h after application. Nevertheless, taking into account that approximately 200 µg/cm² JNJ7777120 was applied topically and 10% are penetrated within 24 h and the concentration gradient decreases exponentially in the epidermis, it can be concluded, that the concentration of JNJ7777120 in the epidermis is in the micromolar range. Referring to an IC₅₀ of JNJ7777120 for the canine H₄R of about 50 nM (17), a reliable inhibitory effect can be postulated.

Atopic lesions after pretreatment with H₄R antagonists following challenge with Df antigen

Twenty-four hours after challenge, placebo-treated animals had a median lesional score of 2. Treatment with topical and oral JNJ28307474 resulted in a median score of 2.5. After topical administration of JNJ7777120, the median lesional score was 2. The triamcinolone solution prevented all dogs from having any lesions (lesional score 0) (Figs 1 and 2).

Atopic lesions after pretreatment with H₁R antagonists following challenge with Df antigen

Placebo-treated dogs showed a median lesional score of 1.5 when examined 24 h after challenge. H₁R antagonists did not significantly reduce the inflammation; cetirizine- and hydroxyzine-treated animals had a median lesional score of 2 (Fig. 3).

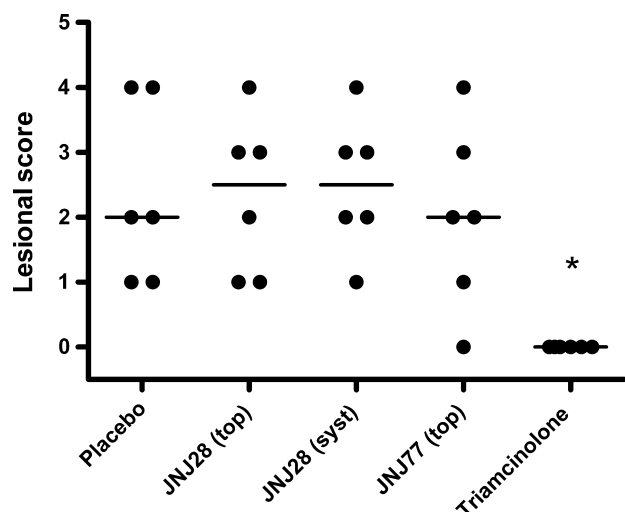


Figure 1. Lesional score in the axillary region of six atopic Maltese-beagle crossbred dogs 24 h after epicutaneous administration of *Dermatophagoides farinae* (Df) antigen. Effects of topically administered JNJ7777120 and topically and systemically administered JNJ28307474 on the lesional score. Topically administered triamcinolone acetate served as a positive control and led to a significant inhibition of the Df-induced lesions, * $P < 0.05$ compared to placebo.

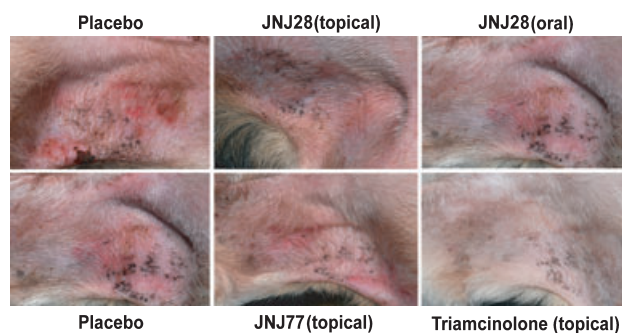


Figure 2. Typical lesions of one dog after treatment with placebo, topically administered JNJ7777120, topically and systemically administered JNJ28307474 and topically administered triamcinolone acetate.

Determination of histamine content in Df-induced atopic skin lesions

By means of microdialysis technique, the amount of secreted histamine in skin challenged with Df allergen was measured in the acute phase and after 6 and after 24 h. Only during the initiation (1–3 h), increased concentrations of dermal histamine were detected (Figure S2). After 24 h, typical acute atopic lesions had occurred at the site of allergen application. However, this obvious inflammatory reaction was not accompanied by an increased secretion of histamine. The concentration of the major allergen Derf 1 was under the limit of detection for most samples. However, 6 h after administration of the allergen, there was a detectable concentration of Derf 1 (approximately 30 pg/ml) in the microdialysate of one dog; this corresponded to a concomitant rise in dermal histamine (Figure S2).

Discussion

Histamine is a well-known and intensively studied mediator in allergic diseases like asthma and AD (22). However, the therapeutic

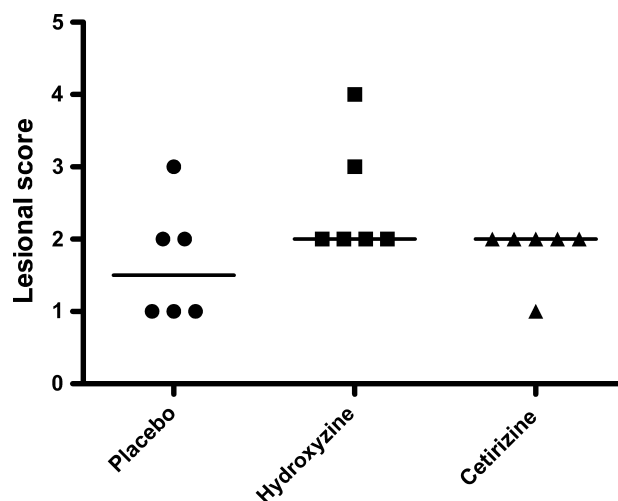


Figure 3. Lesional score in the axillary region of six atopic Maltese-beagle crossbred dogs 24 h after epicutaneous administration of *Dermatophagoides farinae* antigen. Effects of orally administered hydroxyzine (2 mg/kg) and cetirizine (0.5 mg/kg) on the lesional score.

value of classical antihistamines in the control of allergic inflammation in humans as well as atopic dogs is limited (8–10). The lack of efficacy of H1R antihistamines for the treatment of atopic skin lesions might partly be because of the lack of blocking of the H4R-mediated histamine actions. H1R antihistamines (e.g. diphenhydramine, cetirizine, loratadine) do not bind to the H4R (23). Furthermore, the expression profile of the H4R on cells that are directly involved in the pathogenesis and maintenance of AD makes this receptor a promising target for chronic allergic diseases (7). The H4R has been found on dendritic cells (24), particularly Langerhans cells (25), and an increased chemotactic activity via the H4R has been demonstrated for skin dendritic cells (19,25). Additionally, a functionally active H4R has been described in a special subtype of dendritic cells (inflammatory epidermal dendritic cells) that plays an important role in the maintenance of AD (26). A functional H4R has also been described in murine and canine mast cells (27,28) and on Th2 cells (29), which further underlines the possible involvement of this receptor in atopic diseases.

As the first series of H4R selective antagonists displayed anti-inflammatory and antipruritic *in vivo* efficacy (11,12), it was decided to evaluate the highly selective H4R antagonists JNJ7777120 [K_i (canine H4R) = 50 nM (11)] and JNJ28307474 [K_i (canine H4R) = 62 nM (18)] in a canine model of AD, which exhibits several characteristics of acute atopic lesions in dogs (16). In this setting, the preventive administration was chosen as the chance for an inhibitory action of these H4R was estimated higher in a preventive manner compared to a *post hoc* therapeutic intervention. Both antagonists had displayed anti-inflammatory action in an allergic contact dermatitis model in mice induced by the strong hapten FITC (18). The dose and route of administration of JNJ28307474 were directly adapted from this study. For the topical administration, results of the permeation study (Figure S1) were taken as a basis, and it was decided to administer the substance twice 24 h and 60 min before challenge with Df antigen. This protocol should result in micromolar concentrations of the H4R antagonists in the epidermis.

Interestingly, we (12) and others (30) demonstrated that JNJ777120 did not inhibit the allergic inflammatory reaction in acute murine models of allergic dermatitis, which is in line with the observed results in this acute canine model of AD skin lesions. It is thus tempting to examine a possible immunomodulatory action of H₄R antagonists in chronic, but not acute lesions of AD, as Seike et al. (2010) (30) showed the anti-inflammatory action of JNJ777120 in a chronic model of allergic dermatitis provoked by the repetitive administration of the hapten trinitrochlorobenzene.

To assess a possible role of the H₁R in this model of AD, in a second setting, hydroxyzine, which has been used in few small clinical trials in atopic dogs with moderate efficacy (8), and its active metabolite cetirizine were also tested in a preventive manner. Again, no influence on the lesional score was obvious confirming the modest overall efficacy of H₁ antihistamines to prevent the development of lesions of AD, when administered as a monotherapy (8,10). Of particular interest, the selected doses of cetirizine and hydroxyzine have been shown to inhibit histamine- and allergen-induced wheal and flare reactions in dogs (13,14) but they were ineffective in the present model. This might indicate that this model of AD is a more predictive model than intracutaneous tests with anticanine IgE or *Ascaris* antigen.

To gain knowledge about the presence of histamine in the development of acute atopic lesions, microdialysis experiments were performed. Interestingly, only in the induction phase (1–3 h after topical administration of Df allergen), elevated amounts of histamine were found. In late reactions (24 h after challenge), histamine was not detected in high levels, suggesting that it might play only a minor role with other mediators being responsible for the obvious inflammatory reaction.

Can results from this experiment in atopic dogs be translated to humans with AD? As reviewed recently (31,32), the dog is the only species, which lives in an environment similar to that of humans, that spontaneously suffers from naturally occurring AD. Moreover, skin lesions of natural or allergen-provoked AD are very similar to those of humans at pathogenic, clinical and therapeutic levels (31,33). As a result, allergen-induced lesions induced in mite-allergic dogs are a very relevant model to study preclinical treatment modalities.

In conclusion, the administration of H₁R or H₄R antagonists did not prevent the development of acute atopic skin lesions in this canine AD model. By contrast, the glucocorticoid-positive control led to the expected prevention of lesions. However, the limitation of this study is that only the acute phase can be examined in this experimental setting and no anticipations for effects of H₁R and H₄R antagonists on chronic lesions can be made.

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Conflict of interest

This study was supported by Janssen Animal Health, Beerse, Belgium and Johnson & Johnson, La Jolla, USA.

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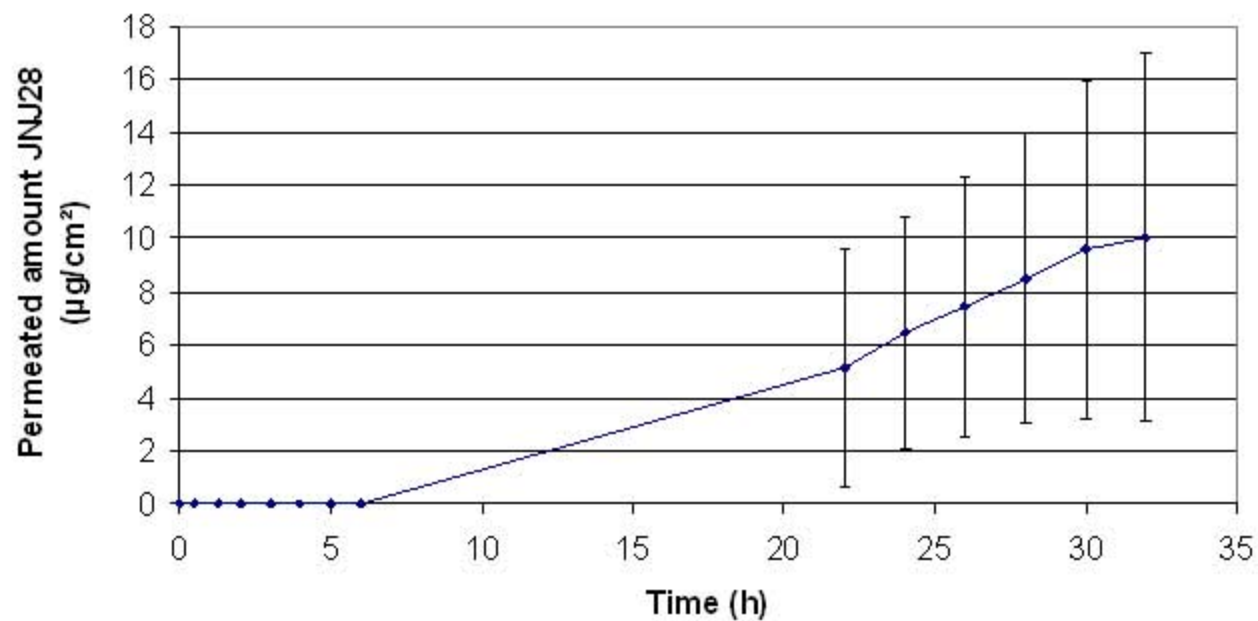
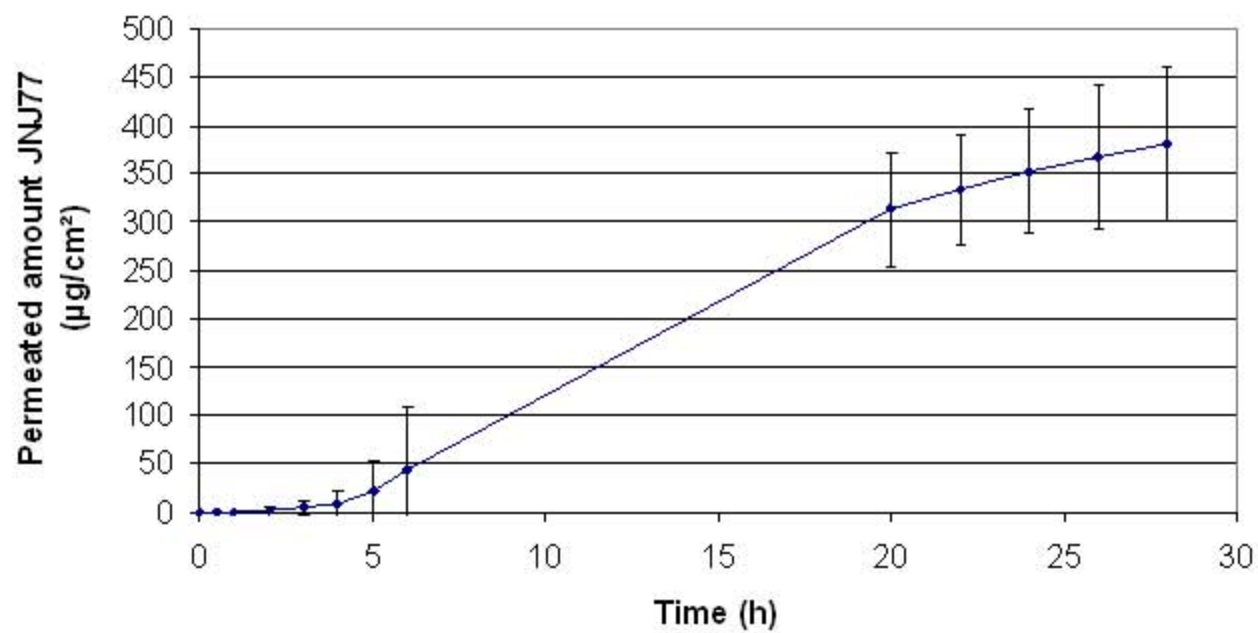
Supporting Information

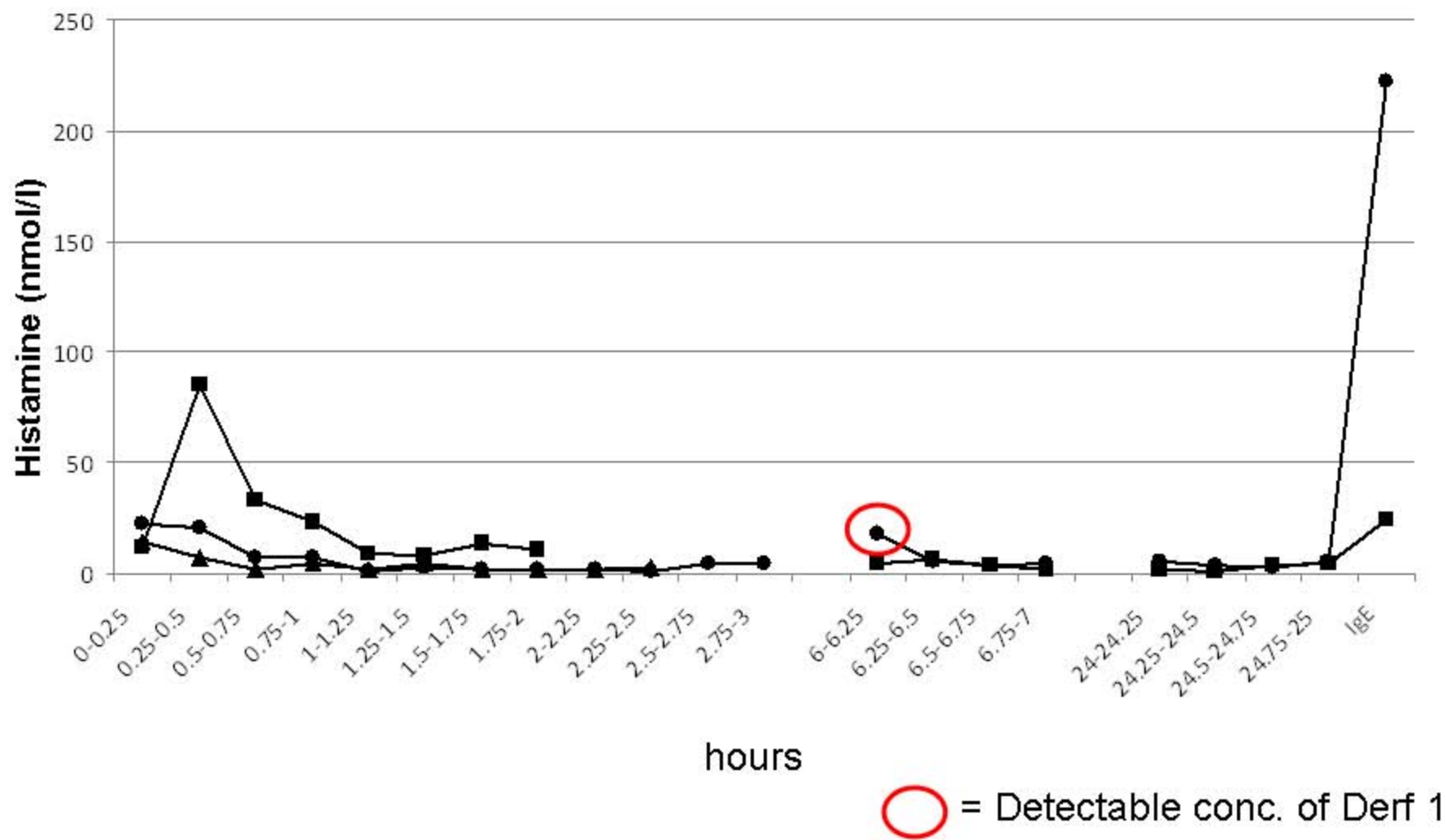
Additional Supporting Information may be found in the online version of this article:

Figure S1. Amount of permeated JNJ777120 (A) and JNJ28307474 (B) in the acceptor medium after topical administration of a 1% formulation on dog skin (mean ± SD of three different skin samples/each experiment).

Figure S2. Concentration of histamine in the microdialysate after epicutaneous administration of Df antigen.

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Supplementary Figure 1: Amount of permeated JNJ7777120 (A) and JNJ28307474 (B) in the acceptor medium after topical administration of a 1% formulation on dog skin (mean $\pm$ SD of 3 different skin samples/each experiment). After 28 h 33.5 % of the administered JNJ7777120 and 0.75% of JNJ28307474 can be found in the acceptor medium.

Supplementary Figure 2: Concentration of histamine in the microdialysate after epicutaneous administration of Df antigen. Individual data from three dogs are shown. Samples were taken for two to three hours after topical administration of the Df allergen. A second and third measurement was performed in two dogs (circle and square) between 6 and 7 hours and between 24 and 25 hours after topical administration of the Df allergen. Only during the first three hours significant amounts of histamine was measured. One dog had slightly elevated histamine at the beginning of the 6 h sampling and detectable Df allergen levels. Intradermal injection of anti IgE (8 μ g), serving as positive control at the end of the study, induced histamine release in 2 of 2 dogs.