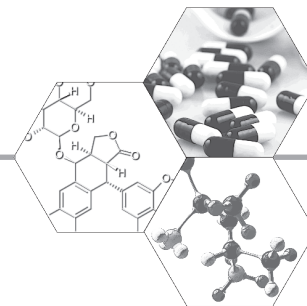




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Nonhuman targets in allergic lung conditions

Existing therapies for allergic asthma are far from perfect: the global prevalence of disease increases despite them and they are poorly effective in dealing with the exacerbations that account for hospitalization and asthma deaths. Commercially, there are pressures on these existing medicines too – a growing threat from generics and reluctance by payers to reimburse for increasingly marginal improvements in medicines with precedented mechanisms. Experience shows that attempts to devise selective small-molecule interventions directed at the myriad of downstream effector pathways has not been a fertile ground for the development of effective new medicines. An alternative strategy, exploiting breakthroughs in understanding the molecular basis of allergenicity and the key role of innate immune mechanisms in asthma, is to direct new approaches to the disease triggers themselves: allergens. This raises interesting possibilities for anti-Lipinski drug design (extracellular nonhuman targets, inhaled delivery) and creates unprecedented pharmacological opportunities in the therapeutic area.

Asthma is generally considered to be the commonest lung disease with an allergic etiology. In approximately 90% of children and 60% of patients overall, it is associated with sensitization to inhaled **allergens**, especially those from **house dust mites** (HDM) [1–3]. Although therapeutic approaches have historically sought single solutions for the treatment of asthma, the fact that it can be evoked by other, nonallergic, triggers reminds us that the condition is not actually a single disease: corollary existing therapeutic approaches may, therefore, not be optimal for many patients.

There is a common perception that modern therapies have rendered asthma a benign condition that is well controlled by effective and inexpensive generic medications. While parts of this perception may be valid, this top-level view conceals some worrisome facts and, on closer examination, it is obvious significant unmet needs remain. Although deaths from acute asthma reactions are comparatively few – approximately 1300 per annum in the UK, 75% of which may be preventable – the socioeconomic burdens of asthma are large: in the UK each year more than 1 million working days lost and the cost to the NHS estimated to be greater than GB£1 billion. Moreover, asthma prevalence continues to rise [4]. This is true across the seven major pharmaceutical markets and, where reliable data exist, it is also true in emerging economies: Brazil already has a high prevalence of asthma and China has a significant number of people with persistent allergic rhinitis, a condition which, in the ‘allergic march’ frequently heralds the development of asthma [4]. Although the underlying

reasons behind these trends are complex, it is hard to escape two conclusions:

- That existing therapeutic approaches are insufficient;
- That increased exposure to factors that result in disease expression must be occurring.

Asthma management is set out in guidelines that describe a stepwise approach to treatment. The standard of care involves the use of β_2 agonists as bronchodilators and inhaled corticosteroids (ICS) as anti-inflammatory agents. Nowadays, these are most effectively employed as combination therapy. Other therapeutic classes, such as antileukotrienes are available for use in patients who are responsive, but the reliance on the tried and tested β_2 agonist/ICS approach is overwhelming. In all but the most extreme steps of asthma management, the β_2 agonist and ICS standard of care is delivered by inhalation. The logic of treating a respiratory disease with an inhaled medicine has become widely accepted, even though the technical requirements of the route are demanding and the need for a delivery device adds significantly to cost of goods. However, it is pertinent to reflect that the elegant site-specific delivery of β_2 agonists and ICS by inhalation is not a requirement of asthma therapy per se, but actually a pragmatic response to limiting their on-target receptor-mediated effects that would be intolerable in chronic systemic therapy.

The objective of modern asthma management is to prevent exacerbations of the disease. There is good reason to avoid these ‘flare-ups’ because they can be hard to control as ICS are less efficacious when they occur. As a result, exacerbations

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Key Terms

Allergens: Antigens, usually proteinaceous, which stimulate a type I hypersensitivity response of the immune system in individuals with a genetic predisposition to allergy.

House dust mites: Tiny creatures, related to spiders and scorpions, with a near-global distribution. Found in human dwellings and are common triggers of allergic asthma because they contain a range of potentially allergenic proteins, among which are the clinically significant Group I allergens.

frequently result in hospitalization and account for most asthma deaths. The reasons for the occurrence of exacerbations are complex, ranging from poor patient compliance to the activity of other predisposing factors such as alterations in allergen exposure and the effects of infections, especially certain respiratory viruses.

The failure of existing medicines to reduce the burden of asthma indicates a need for improved therapies, but where will they come from? Over the past 30 years, asthma drug discovery has been predicated on the flawed assumption that better treatments would arise inexorably from a greater understanding of its pathobiology. However, the pharmaceutical industry failed to appreciate that increasing knowledge of complex biological mechanisms tells us more about the resilience of networks and that, paradoxically, the identification of ever more targets can indicate a diminishing probability of drug-discovery success. Thus, despite 40 years of intensive effort, attempts to discover new small-molecule anti-asthma medicines have enjoyed little success. Alternative bronchodilators have had no obvious benefits over existing approaches and selective antagonists directed at a range of inflammatory mediators have failed to offer a viable alternative to ICS. With the exception of antileukotrienes, which are efficacious in a minority of patients, none of the small-molecule approaches directed at specific mediators have delivered significant new asthma medicines to the market. Failure has mainly occurred because of poor efficacy; the hard-learned and expensive lesson being that the complex and highly orchestrated immuno-inflammatory responses of asthma are backed-up by multiply redundant pathways that make it difficult for single mediator interventions to be successful. ICS work because of their broad spectrum of actions but the penalty paid is an extensive palette of adverse effects.

What then for the future of asthma therapy? Small-molecule interventions can be broadly categorized into those exploiting precedented mechanisms and those pursuing novel targets. Precedented mechanisms encompass iterations of the β_2 -agonist/ICS approach, and this remains an area of active endeavor. To be commercially successful, these must offer significant advantages over existing products but this feat is becoming harder to achieve in ways that ensure premium reimbursement prospects. For drugs targeting unprecedented mechanisms, the measure of success is whether the approach will offer significant advantages over the existing

– often effective but imperfect – standard of care. History teaches that the prospects for success from other interventions targeting discrete low-molecular-weight inflammatory mediators are likely to be limited, but immunomodulation offers an interesting possibility. If the probability of success for small-molecule interventions against ‘classical’ inflammatory mediators is low, then other targets such as IgE, cytokines and chemokines may be more successful. This is the strategy behind a number of programs in clinical development where asthma/allergy-related targets (e.g., IL-5, IL-13) are being addressed using monoclonal antibodies. By their nature, biologics suffer from a number of disadvantages that may restrict their application to severe asthma that is unmanageable by other approaches. The pharmaceutical dilemma this creates is demonstrated by the marketed anti-IgE therapy omalizumab (XolairTM). Omalizumab depletes circulating IgE, with the assumption this eventually diminishes the amount of IgE bound to mast cells to a level capable of attenuating allergic responses. Outside the USA, its use is confined to severe asthma because of its high cost and marginal benefit (>£30,000 per quality-adjusted life year gained) [5]. In England and Wales NICE restricts the use of omalizumab to patients who have demonstrated a history of treatment compliance, thereby effectively shrinking its market potential to a small niche, because poor compliance is a major contributory factor to serial asthma exacerbations. In the absence of stellar efficacy it is probable that similar handicaps will apply to other high cost anti-asthma biologics currently in development. Despite this, and because reimbursement policies differ by territory, omalizumab achieves significant global sales (~US\$1 billion in 2011). However, even in these territories it is reasonable to speculate that reimbursement policies will become increasingly pernicious, creating a significant threat to high cost biologics in the longer term.

With the exception of omalizumab, whose rationale heralded a philosophical shift from treating asthma symptoms to dealing with its precipitating factors, the feature common to the small molecule or biologics approaches outlined above is that they target complex downstream events that are triggered by the stimuli that cause an asthma attack. Clinically and commercially, this method of approaching asthma therapy is in danger of fast becoming a spent force for innovation and patient benefit.

Targeting triggers of asthma

Recent progress provides new opportunities for anti-asthma drug discovery that are based on attacking the triggers of disease, rather than managing their consequences. The scientific advances that create this possibility are: first, an improved understanding of the molecular basis of allergenicity, and second, a definition of the innate immune mechanisms that have a central role in orchestrating the development and maintenance of allergy.

The risk factors for allergic asthma are well established. HDM are the major cause of domestic allergies worldwide and their avoidance, although hard to implement effectively, brings significant clinical improvement. They trigger asthma attacks because they are sources of proteins (allergens) which potently stimulate the production of IgE to cause allergic sensitization. After sensitization, further exposure to the allergens elicits the IgE-dependent release of mediators that trigger the contraction of airway smooth muscle and promote inflammation. Nowadays, definitions of the terms allergy and allergen are habitually associated with the production of IgE, but as we understand more about the roles of innate signaling in the responses to allergens, it is clear that IgE production is but one of many effector pathways associated with these foreign proteins. Thus, the overarching biological response to (at least some) allergens involves both IgE-independent and IgE-dependent effects. Whilst the former are required to initiate and maintain sensitization, they occur alongside IgE-dependent actions and form part of the integrated response to inhaled allergens. It is becoming apparent that the IgE-independent component could be of substantial significance, perhaps requiring that the modern definitions of allergy and allergens come full circle and return to the mechanism-neutral definitions originally coined by Clemens von Pirquet in 1906 [6].

HDM exemplify how increased allergen exposure accounts for the rise in asthma prevalence [7]. A succession of epidemiological studies shows that sensitization to HDM results in the expression of allergic diseases, including asthma [1,8–17]. Allergy to HDM results from sensitization to allergens that are present largely in the fecal pellets excreted by these tiny creatures [18]. These proteins are not equally allergenic; their differing allergenicity depends upon their functional activity, which is now recognized as providing a crucial bridge between innate and acquired

immunity through, among others, the activation of pattern recognition receptors (PRRs). Cell surface PRRs include P2Y₂ (for sensing ATP released following allergen exposure), Toll-like receptors (TLRs) and C-type lectin receptors (CTLRs). All have a role to play in the overall response to the gamut of HDM allergens and, in the case of TLRs and CTLRs, the recognition event depends on a feature that is associated with the function of the allergen in HDM.

However, not all allergens perform this bridging role effectively, in fact only a small cadre appear to do this really well. Allergens with peptidase activity, which are notably represented in HDM, are prominent members of this small cadre because their peptidase activity triggers a triad of events (allergen delivery, effector cell recruitment and the breaking of immune tolerance) that promotes sensitization to HDM allergens and, significantly, to bystander allergens – regardless of source – encountered at the same time. In conjunction with the activation of other innate mechanisms by other allergens, this creates a potent stimulus for the development of allergy, especially in those with a genetic predisposition. The bystander interaction is interesting since it provides a mechanism for multiple sensitization and an opportunity for therapeutic intervention. To exploit any opportunity of intervention at the trigger level, it is necessary to understand the potential target(s) in more detail.

Mite allergens

Various mites are associated with allergic disease, the clinically significant families being Pyroglyphidae (e.g., *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Euroglyphus maynei*), Acaridae (e.g., *Acarus siro*, *Tyrophagus putrescentiae*, *Tyrophagus longior*), Glycyphagidae (e.g., *Glycyphagus domesticus*, *Lepidoglyphus destructor*) and Echimyopodidae (e.g., *Blomia tropicalis*) [18]. Of these, mites of the genus *Dermatophagoides* are globally the most important, and although *D. pteronyssinus* probably has the wider geographical distribution, *D. farinae* is of note because it is able to thrive in drier climatic conditions [19]: warmth and especially humidity are critical factors for mite growth.

Causes of allergy are almost invariably associated with multiple allergens and as can be seen from **TABLE 1**, the allergenic armory of mites is extensive. Allergens from different species within a genus, or across phylogenetically related genera but similar on the basis of sequence identity, are grouped together. Thus,

Key Term

Der p 1: Example of the Group I allergens from house dust mites. These are of major clinical significance in allergic asthma. Group I house dust mite allergens are cysteine peptidase digestive enzymes that become excreted in the fecal pellets of mites. These droppings are of respirable size for humans and when they impact upon the airway lining their contents are released, triggering responses via innate and allergic immune mechanisms in genetically predisposed individuals.

Table 1. Physicochemical and biochemical features of mite-derived allergens.

Allergen	Frequency of immunoreactivity	Molecular weight (kDa)	Function
Group 1 (e.g., Der p 1)	>90	25	Cysteine peptidase
Group 2 (e.g., Der p 2)	>90	14	MD-2 related protein
Group 3 (e.g., Der p 3)	90	25	Trypsin
Group 4 (e.g., Der p 4)	25–46	60	Amylase
Group 5 (e.g., Der p 5)	9–70	14	Function unknown; possible ligand binding protein
Group 6 (e.g., Der p 6)	39	25	Chymotrypsin
Group 7 (e.g., Der p 7)	53–62	26–31	Function unknown; possible pathogen associated molecular pattern binding protein
Group 8 (e.g., Der p 8)	40	27	Glutathione-S-transferase
Group 9 (e.g., Der p 9)	>90	29	Collagenase-like serine peptidase
Group 10 (e.g., Der p 10)	81	36	Tropomyosin
Group 11 (e.g., Der f 11)	82	103	Paramyosin
Group 12 (e.g., Blo t 12)	50	16	May be a chitinase; shows homology with Der f 15
Group 13 (e.g., Lep d 13)	11–23	15	Fatty acid binding protein
Group 14 (e.g., Der f 14)	84	177	Vitellogenin or lipophorin
Group 15 (e.g., Der f 15)	95	63/98	Chitinase; shows homology with Blot 12 allergen
Group 16 (e.g., Der f 16)	50–62	53	Gelsolin
Group 17 (e.g., Der f 17)	35	30	Calcium binding protein
Group 18 (e.g., Der f 18)	63	60	Chitinase
Group 19 (e.g., Blo t 19)	10	7	Antimicrobial peptide homology
Group 20 (e.g., Der p 20)			Arginine kinase
Group 21 (e.g., Der p 21)	26	15	Function unknown; shows homology with Group 5 allergens
Group 22 (e.g., Der p 22)	ND	ND	Shows homology with Group 2 mite allergen, belongs to ML domain family, implicated in lipid binding
Group 23 (e.g., Der p 23)	ND	14	Unknown function, shows homology with peritrophin-A domain
Group 24 (e.g., Tyr p 24)	11	18	Troponin C, shows homology with Bla g 6

ND: Not determined.

the Group 1 HDM allergens comprise the proteins **Der p 1**, Der f 1, Eur m 1 and Blo t 1 and these are similar on the basis of their amino acid sequences and immunoreactivity [18].

The major allergens from different mite species include enzymes, particularly peptidases involved in digestion, actin-associated proteins (e.g., tropomyosin, troponin C and paramyosin) and ligand-binding proteins [18]. Group 2 allergens, which are clinically important, are found in the gut of mites and are mechanistically interesting because they possess an MD-2 related domain that is capable of binding w-4 [20].

Coupled with the high clinical reactivity of mite-allergic individuals to Group 1 HDM allergens, the discovery that these allergens show sequence identity with cysteine peptidases prompted speculation that this bioactivity may be relevant to their allergenicity. There is well-documented precedent to believe that peptidases are especially potent triggers of asthma. In the 1960s, despite industrial health and safety countermeasures that were notably good for the period, the advent of the first biological washing powders – that used peptidases and other enzymes in their formulation – was associated with occupational sensitization of

workers handling bulk raw materials and of both workers and some consumers handling the finished product. With improved materials handling and effective encapsulation of the enzyme, 'detergent-induced' asthma has been largely eliminated [21].

Therapeutic opportunity

Is it feasible to intervene effectively at the level of a disease trigger in asthma? Avoidance of domestic aeroallergens is one nonpharmacological option that has been actively investigated. Studies in high altitude sanatoria that provide a cold, dry environment not conducive to HDM survival, or in cleanroom environments that exclude HDM, show that allergen avoidance brings significant clinical benefits [22–28]. Unfortunately, stringent conditions are required to achieve these benefits and no matter how diligent the measures taken to ensure allergen-free personal environments, they are negated by there being no hiding place from allergens in everyday life so, disappointingly, allergen avoidance is not generally effective.

What if it were possible to provide individualized protection against aeroallergens which, unlike physical avoidance, would travel with an individual by being delivered to the airways? An inhaled treatment directed at a nonhuman target is a novel approach to asthma, but initially appears a daunting challenge: there are multiple groups of HDM allergens, and polysensitization to allergens from multiple sources is common. However, allergens from the same source clearly differ from each other in abundance and immunoreactivity. Furthermore, the growing understanding of how HDM (and other allergens) activate the innate immune mechanisms that initiate and maintain allergy provides evidence that some allergens are more significant than others. This reduces the scale of the drug design challenge to a point where it becomes viable to examine whether the selective targeting of a key HDM allergen is able to attenuate the response to a mixture of allergens. There are several factors that make such an approach potentially attractive. The nonhuman nature of the target might relax some chemical design issues surrounding selectivity and the fact that it is extracellular means that cellular permeability is unimportant. Indeed, lack of cellular permeability is positively virtuous as it potentially benefits both selectivity and dosing considerations, creating an opportunity to use an approach that is antithetical to Lipinski design principles of

'drug likeness'. To ensure satisfactory systemic absorption of orally delivered drugs, there is a chemical design benefit for small lipophilic molecules (<500 Da) with relatively inflexible scaffolds. For delivery by inhalation, where inevitably a portion of the dose is swallowed, there is benefit in minimizing oral absorption to reduce the potential for systemic adverse events. However, there are practical limits to breaking the Lipinski rules that are not just related to the cost of carbon atoms. Drugs purposed for use in, especially dry powder, inhalation devices require stringent physicochemical behavior that can become harder to manage as molecular weight is increased.

Based upon the effects of Group 1 HDM allergens and their significance in allergic asthma, we embarked upon a program to explore the biology of Group 1 HDM allergens and to conduct a structure-based chemical design campaign to produce inhibitors of them. The intention was that this would ultimately provide the ability to test whether this type of upstream intervention was therapeutically viable.

Structure & catalytic properties of Group 1 HDM allergens

Initial attempts to understand the structure of Der p 1 as a model for other Group 1 HDM allergens were based upon homology modelling against papain, actinidin [29] and cathepsin K [ROBINSON C, ZHANG J, NEWTON GK *ET AL*, UNPUBLISHED DATA]. High-resolution crystal structures have now been obtained for Der p 1 and Der f 1 in both recombinant and native forms [30–32]. Inspection of the crystal structures shows that homology modelling was broadly correct for the catalytic site, although overall there are some notable differences: an unpredicted disulphide bridge between Cys84 and Cys197, metal binding and possible dimer formation. A further difference is the location of Tyr249, which forms part of the boundary of the S4 pocket in the crystal structure, but which forms part of the upper boundary of the S2' pocket in the homology model derived from cathepsin K. The monomeric structure of the dust mite allergens consists of two globular domains separated by a cleft that binds the substrate. The residues around the substrate binding cleft are well conserved, suggesting that substrate specificity is conserved among isoallergens in a single species and homologous allergens between mite species (FIGURE 1). This is borne out by similarities in substrate and inhibitor kinetics [ZHANG J, UNPUBLISHED DATA]. The

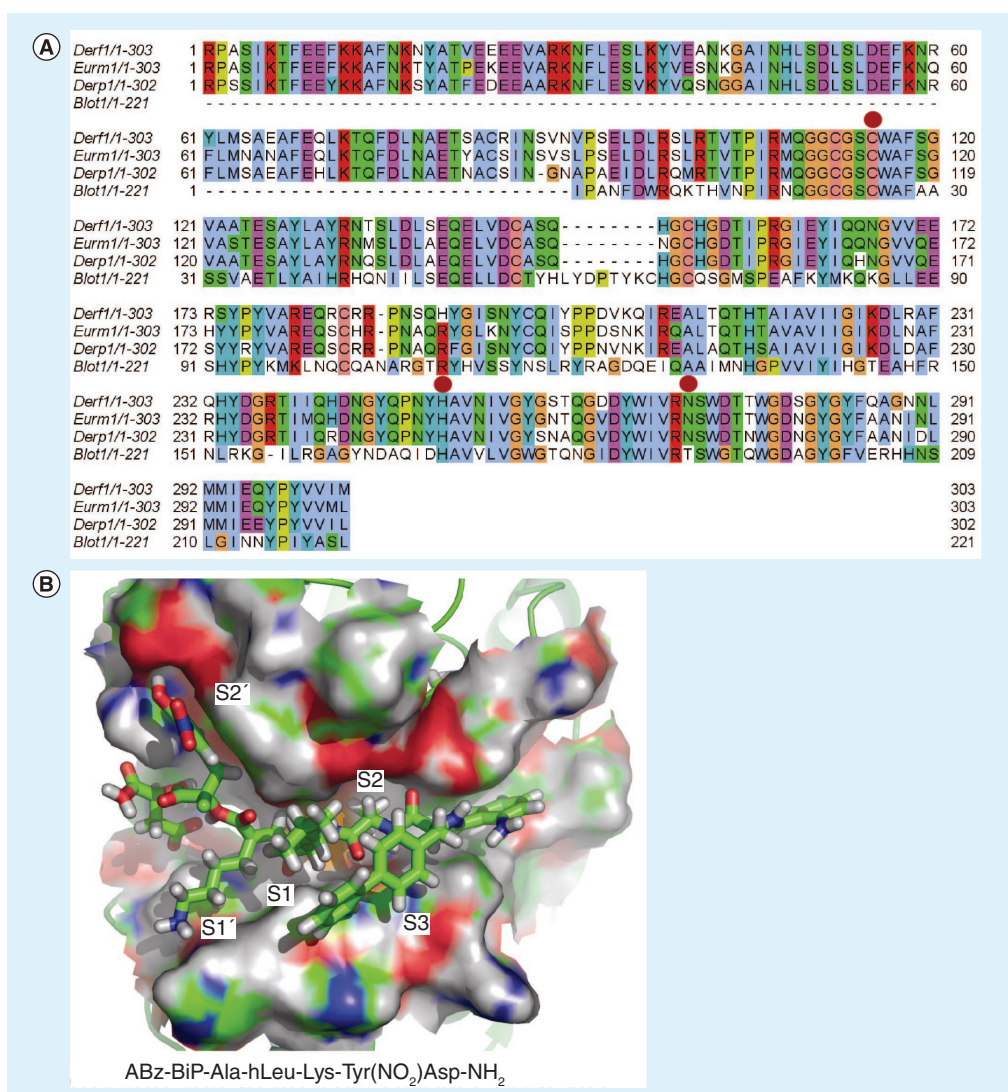


Figure 1. The proteolytic activity of Group 1 HDM allergens constitutes a single drug discovery target as indicated by sequence similarity and substrate preference.

(A) Similarity of Group 1 mite allergens as shown by multiple sequence alignment. Residues are coloured according to similarity of properties (green: polar; purple: acidic; red: basic; dark blue: hydrophobic; light blue: large aromatic polar; and orange/yellow: secondary-structure breaking). All other similar residues are coloured identically. Red markers show location of catalytic site residues (Cys114, His250 and Asn270 in Der p 1). **(B)** Docking of synthetic substrate 2-amino benzoyl-biphenylalanine-Ala-hLeu-Lys-Tyr(NO₂)Asp-NH₂ into the catalytic site of a homology model of Der p 1.

allergens contain three disulphide bridges (in Der p 1 these are Cys84-Cys197, Cys111-Cys151 and Cys145-Cys183).

Production of correctly folded recombinant Group 1 allergens in bacterial expression systems have been frustrated by technical problems and success was not finally achieved until Der p 1 was expressed in a *Pichia pastoris* expression system. However, protein expression in yeast is frequently associated with hyperglycosylation and mutation of glycosylation sites is necessary to prevent this

(e.g., variants N132Q [31] and N132E [30]). Despite this, kinetic studies have shown that native Der p 1 (nDer p 1) behaves similarly to hyperglycosylated recombinant Der p 1 (rDer p 1), indicating that glycosylation is independent of catalytic function [33], although differences in behavior towards PRRs may be anticipated.

The active site of Der p 1 (and analogously Der f 1) with the catalytic triad formed from Cys, His and Asn residues is situated in the cleft between two globular domains. This structure

is typical of cysteine peptidases of clan CA and family C1. The active sites of Der p 1 and Der f 1 are similar (**FIGURE 1**), the only remarkable difference being Gln232 in Der f 1 compared with Arg231 in Der p 1 that might account for the apparently selective inhibition of Der f 1 by a cystatin from chestnut [34].

The Group 1 HDM allergens are initially synthesized in pre-pro forms. With the propiece intact, the protein lacks catalytic activity because the 80 residue propeptide folds into the active site cleft, occluding it [30]. Crossed immunoelectrophoresis and IgE inhibition studies indicate that several epitopes are covered by the propiece [30], providing further confirmation that the catalytically inactive form of Der p 1 is less immunogenic than the mature peptidase [35]. The 4 α -helix, 80 residue propieces of Group 1 HDM allergens are unusual compared those of cathepsins L, S and K which are longer, comprise 3 α -helices and contain a conserved EX₃RX₂(I/V)FX₂NX₃IX₃N or 'ERFNIN' motif [36]. However, the propieces of some cathepsins are shorter than in the Group 1 HDM allergens because they lack the first α -helix (e.g., cathepsin B has a 62 residue prodomain). The Group 1 allergen propieces are therefore of intermediate length and different in structure to other well-characterized cysteine peptidases. These differences affect function: The prodomains of cathepsins are potent inhibitors of their cognate enzymes even when their covalent attachment to the enzyme has been cleaved and this persistence of inhibition by untethered prodomains is thought to provide fine regulatory control over proteolysis. In contrast, the Der p 1 propiece, whose removal is accelerated by active Der p 1, is then rapidly degraded by the mature peptidase [36]. These observations suggest that the Group 1 HDM allergens form a new sub-family of cysteine peptidases and that the rapid degradation of their inhibitory propiece upon maturation may contribute to the notable potency of these allergens.

Elucidation of the crystal structure of Der p 1 indicates the presence of a metal binding site which chelates Mg²⁺ [30,31,37] or Ca²⁺ [32], but bound metal ions were absent in mature native Der f 1, despite conservation of the relevant amino acids [32]. Although divalent cations are known to increase the catalytic activity of papain [32], the binding of metal ions has no apparent effect on the structures of Der p 1 and Der f 1, and their removal by EDTA has no effect on Der p 1 catalytic activity [ZHANG J, UNPUBLISHED DATA].

The ability of recombinant mature Der p 1 to oligomerize in solution and/or in crystal form has been reported [31,32], although native mature Der f 1 is monomeric in both solution and solid states [32]. It is unclear whether the dimeric/multimeric form of Der p 1 occurs physiologically and whether this affects catalytic activity [31,32]. If this were to be a physiological occurrence it would distinguish Der p 1 from most other cysteine peptidases as only cathepsins C is regarded as a physiologically relevant oligomer [38]. However, as noted earlier, other aspects of Group 1 HDM allergen structure–function place them into a distinct sub-family of cysteine peptidases [36], so other differentiating features may be anticipated.

A previous report suggested that Der p 1, in addition to its cysteine peptidase activity, was a serine peptidase [39]. Definitive evidence shows that this claim is erroneous, as demonstrated by both examination of structure [30] and detailed enzymatic analysis of native and recombinant forms of Der p 1 using novel selective inhibitors and substrates [33]. Unlike chymotrypsin, native Der p 1 prepared by rigorous purification fails to degrade *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. The action of chymotrypsin on this substrate is not blocked by ADZ 50,000 (designed as a highly thiol-reactive and selective inhibitor of Der p 1), confirming its design selectivity. In contrast, trypsin and native Der p 1 both degrade *N*-Bz-Phe-Val-Arg-*p*-nitroanilide, but whereas the action of Der p 1 is blocked by ADZ 50,000, it is not affected by the broad-spectrum serine peptidase inhibitor AEBSF [33]. It seems likely that the erroneous claim that Der p 1 has combined cysteine and serine peptidase activity originates from inadequate purification and contamination with Der p 3, Der p 6 and Der p 9, all of these allergens being serine peptidases that require removal by scrupulous affinity chromatography [18]. For the unwary, peptidase carry-over can be a significant problem when working with any purified allergens, regardless of source [ZHANG J, UNPUBLISHED DATA].

Despite these similarities, some differences between the Group 1 allergens from different species of HDM have been noted that affect their epitopes [32]. These are believed to explain the specificity and the cross-reactivity of antibodies against Der p 1 and Der f 1; factors that have a potential bearing on the engineering of hypoallergenic variants of these proteins for use in allergen-specific immunotherapy applications.

Biological effects of Group 1 HDM allergens

The sequencing of Der p 1 suggesting that it and, by inference Group 1 HDM allergens generally, might be a functional cysteine peptidase was followed by a demonstration of its catalytic competence. This prompted investigations into whether its proteolytic activity contributed to allergy. Given the rationale that inhaled allergens impact upon the airway mucosa, the effects of Der p 1 on the airway epithelium was first examined and demonstrated an increase in epithelial permeability. At high concentrations this effect occurs through epithelial cell loss [40,41], but subsequent studies using more sensitive approaches revealed that at lower exposure levels, subtle and localized effects occurred through the cleavage of tight junctions (TJs) [42,43]. TJs are the adhesion complexes that protect the entry to paracellular channels in the airway epithelium and, which therefore regulate the extracellular transepithelial disposition of materials deposited upon the airway mucosa. Airway surface lining fluid contains a number of natural peptidase inhibitors, notably the serpin α_1 -antitrypsin which is inactivated by Der p 1 [44,45]. However, Der p 1 itself is resistant to inhibition by most natural antipeptidases: of mammalian antipeptidases examined, α_2 -macroglobulin is the only notable inhibitor and this is only found in low abundance in airway surface liquid [46]. Der p 1 also cleaves surfactant proteins A and D, soluble pattern-recognition receptors of the Collectin CTLR superfamily, which are believed to exert protection against allergy [47].

The number of interesting and potentially relevant targets of Group 1 HDM allergens continues to grow, suggesting that the influence of proteolytic activity upon the initiation and maintenance of allergic sensitization could be extensive. A range of potential effects is shown in **TABLE 2**.

The cleavage of TJs and the opening of paracellular routes in the airway epithelium are of particular interest in the initiation and maintenance of allergic sensitization, as well as the responses to inhaled allergens. First, it provides a mechanism that increases the probability of detection of an allergen by dendritic antigen presenting cells normally resident beneath the airway epithelium. Because the increase in permeability resulting from TJ cleavage is nonselective it would enhance the delivery of any allergen to dendritic antigen-presenting cells. Second, it should facilitate the MD-2 co-receptor surrogacy of the Group 2 HDM allergens, driving their interaction with TLR-4, which has a mainly basolateral distribution (i.e., protected by TJs and the epithelial permeability barrier) in the airway epithelium [48]. By the criteria of molecular weight and size, Group 2 HDM allergens are significantly different and so their permeability would be expected to increase to a greater degree as TJs are cleaved. This may contribute to the high level of immunoreactivity of patients to this allergen. Third, the opening of paracellular pathways provides a migration route for activated DCs and other cells to enter the airway lumen and, if required, return.

The spectrum of peptidase actions in the airway mucosa extends beyond an influence over the epithelial disposition of inhaled allergens. In allergic asthma there is an increased number of DCs in the airway mucosa [49–51]. The recruitment of dendritic cells by Der p 1 is fostered by CCL-2, CCL-20, CCL-5 and CXCL-10 released by asthmatic airways [52]. Of these, CCL-20 may be particularly significant because it signals preferentially through CCR-6, which is expressed on DCs that accumulate at inflamed mucosal sites. There is compelling evidence that the epithelial-DC axis is central in orchestrating the evolution of experimental allergic responses, especially the recruitment of eosinophils [53]. In mice deficient in CCR6, pulmonary responses to allergens are attenuated as a consequence of the inhibition of DC migration [54]. The presence of DCs beneath the airway epithelium, and in increased numbers in the airway lumen following allergen challenge, suggests that some allergens have a key role in

Table 2. Allergy-related biological effects of house dust mite peptidase allergens.

Effect	Allergen	Ref.
Cleavage of tight junctions/allergen delivery	HDM fecal pellet extract, Der p 1, Der p 3, Der p 6, Der p 9	[40,42,43,91,92]
Cleavage of low affinity IgE receptor (CD23)	Der p 1	[67]
Cleavage of DC-SIGN/R	Der p 1	[55]
Cleavage of IL-2R (CD25)	Der p 1	[66]
Inactivation of surfactant proteins	Der p 1, Der f 1	[47]
Inactivation of airway antiproteases	Der p 1	[44,45]
Cytokine/chemokine expression and release	HDM extract, Der p 1, Der p 3, Der p 6	[52,57–60,69,93,94]
PAR-2 activation	Der p 3, Der p 1,	[58,59,69]
PAR-1 inactivation	Der p 1	[58]
Breakdown of immune tolerance	HDM extract, Der p 1	[76,95,96]
IgE-independent mast cell stimulation	Der p 1, HDM extract	[60]

their recruitment, although the precise mechanisms await definition. The C-type lectins DC-SIGN and DC-SIGNR have been proposed as putative substrates of Der p 1 [55], although there is doubt about whether the observed changes in DC-SIGN expression following exposure to Der p 1 are due to proteolysis or not [55,56].

Pertinent to the activation of innate responses leading to the development of allergy, HDM peptidase allergens – especially Der p 1 – have the capability to release or activate pro-inflammatory mediators (eicosanoids, cytokines and chemokines, histamine and complement) [57–60]. Combined with an ability to inactivate mucosal defences, principally through inactivation of protective antiproteases that may already be intrinsically defective in allergy through genetic polymorphism [61–65], this creates an environment that favors the development of disease. Additional receptor cleavage events, of which there are several notable examples suggested by *in vitro* experiments, would serve to reinforce this tendency. Cleavage of the α subunit of the IL-2 receptor (CD25) on T cells would shift the balance of immune responses to a Th2 phenotype [66], and cleavage of the low affinity IgE receptor (Fc ϵ R2, CD23) on B cells [67] would impair regulatory control over IgE synthesis. Protease-activated receptors (PARs), whose expression is up-regulated in asthma [68], are also potential targets of HDM peptidase allergens and have been proposed as the receptors initiating the release of IL-6, IL-8, GM-CSF, CCL-5 (RANTES), and CCL-11 (eotaxin-1) from epithelial cells [58,59,69]. PARs are characteristically activated by serine peptidases and functional evidence supports their cleavage and activation by Der p 3 and Der p 6, which are trypsin-like and chymotrypsin-like HDM peptidase allergens respectively [59,69]. Although widely assumed, it is less clear whether PAR cleavage by Group 1 HDM allergens activates signal transduction and mediator release [58], and it is conceivable that the response to HDM allergens in real-life may actually inactivate PAR signaling as a result of nullifying cleavages of PARs by the dominant presence of Group 1 allergens.

Substrate specificity & inhibitor design

Few surveys of the substrate specificity of Group 1 HDM allergens have been undertaken, in part reflecting the difficulty of obtaining suitable amounts of native allergens to support a comprehensive screening campaign and, until

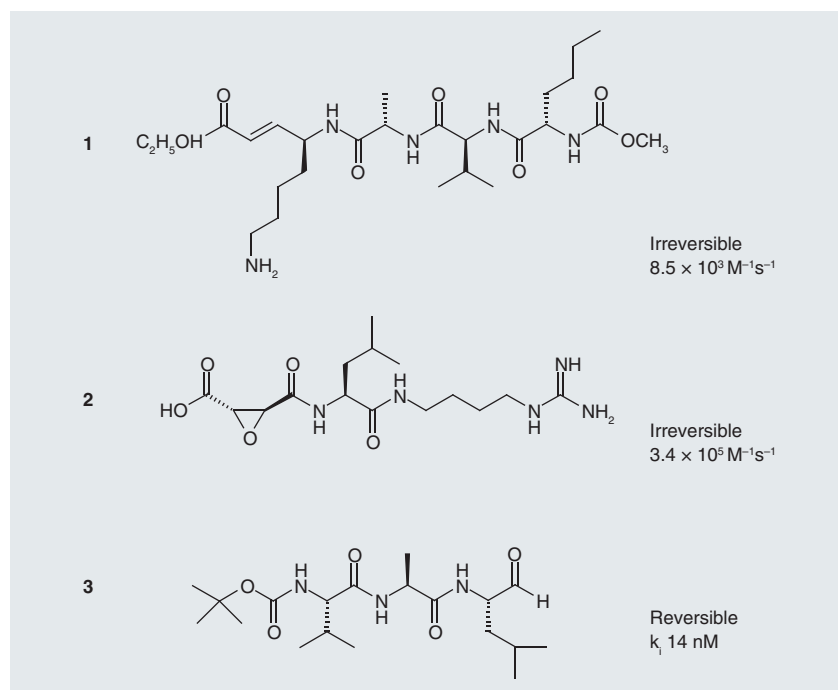


Figure 2. Inhibitors of Der p 1 (1,3) and comparison with generic cysteine peptidase inhibitor E-64 (2).

recently, technical hurdles in the recombinant expression of these allergens in a correctly folded, catalytically active form. **FIGURE 1B** shows docking of an experimentally optimized artificial substrate into the active site of Der p 1, which was used to rationalize how occupancy of the

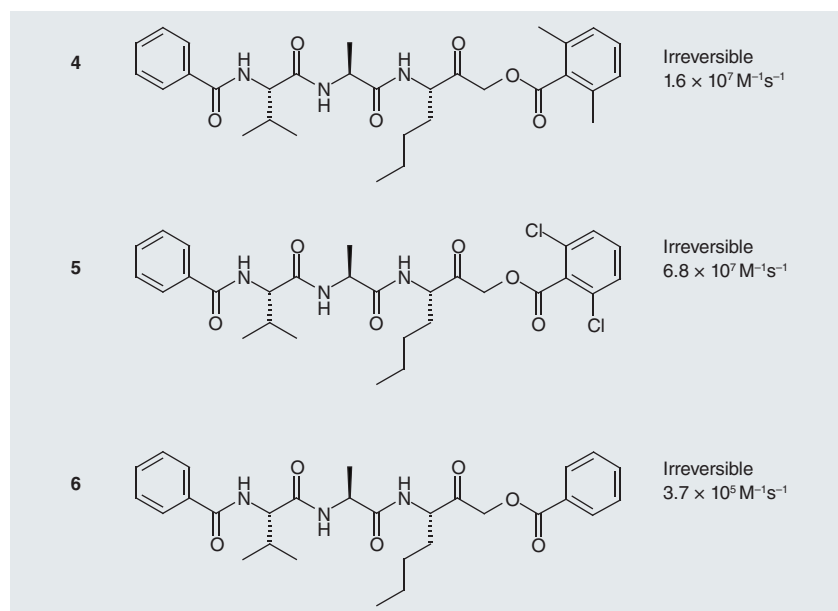


Figure 3. Acyloxymethyl ketone inhibitors of Group 1 mite allergens. These were produced by systematic modification of an optimized substrate revealed by peptide library screening.

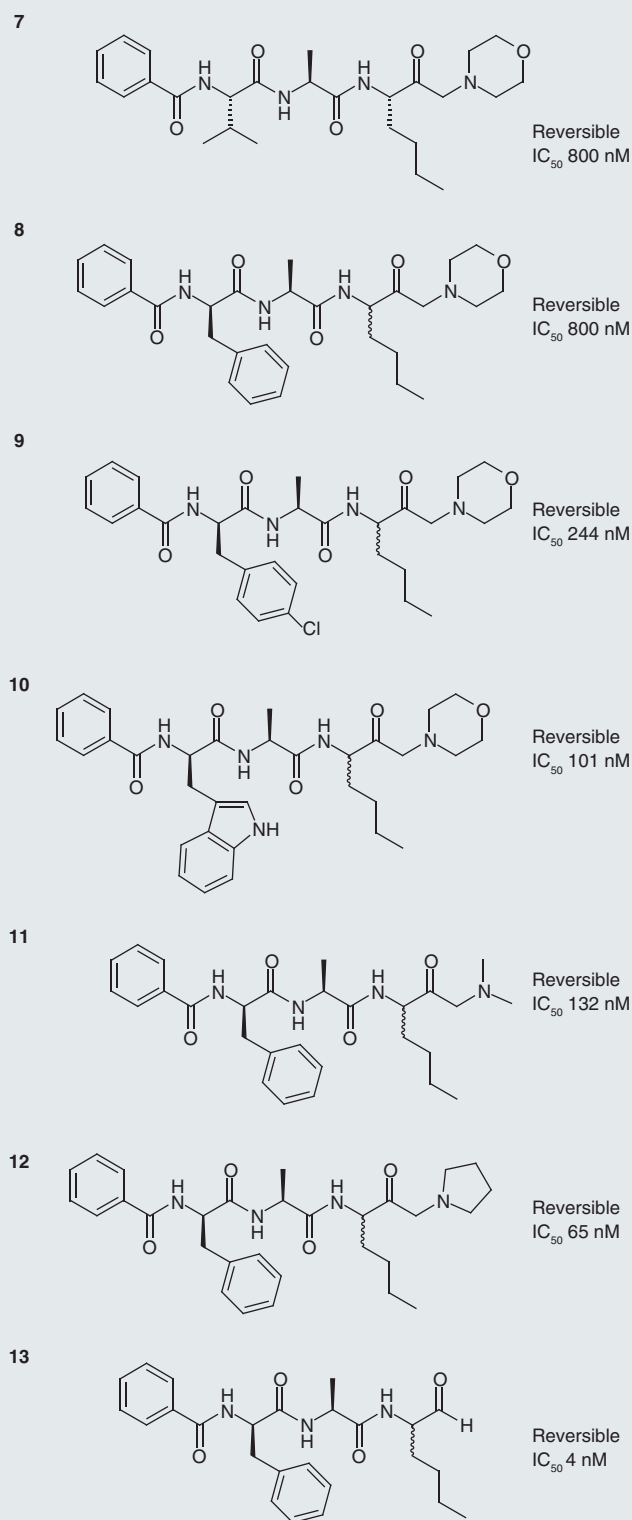


Figure 4. Representative amino ketone inhibitors of Group 1 mite allergens used in SAR exploration.

left predicts substrate preferences in a binding model. Screening a library of FRET substrates of the general formula $Abz-B-C-D-E-Tyr(NO_2)-Asp-NH_2$, with cleavage between D and E, indicated that Der p 1 has preferences for the following B: Val>Ala, Gln, Leu, Phe; C: Ala>>Gln or Lys; D: Leu, Nle or Ala>Ser; E: Ser. Nle being an isostere of Lys, this order is similar to the preferences (P_1 Lys, P_2 Ala, P_3 Val, P_4 Nle) established by screening crude HDM extracts against a library of 137,180 tetrapeptide fluorogenic substrates and then a peptide nucleic acid encoded library of 4000 potential irreversible inhibitors [70].

Mapping of substrate specificity provides one rapid means to identify targeting motifs that may spawn useful scaffolds for drug design. Screening of the inhibitors in the peptide nucleic acid encoded library revealed irreversible inhibitor **1** (FIGURE 2), which has a second order inhibitor rate constant of $8.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ against Der p 1 [70]. However, using a targeting motif does not always lead to potent and selective inhibitors of cysteine peptidases and of itself provides little drug-likeness [71]. This can be seen in the case of Der p 1 by comparing **1** with the generic cysteine peptidase inhibitor E-64 (**2**, FIGURE 2), which is an unremarkable inhibitor of Der p 1 [70]. The latter has a second order inhibitor rate constant of $3.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ [ZHANG, J, UNPUBLISHED DATA], whereas the corresponding constant for ADZ 50,000, a highly reactive Der p 1 inhibitor, is approximately $1.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ [33]. However, despite some promising attributes, ADZ 50,000 is unsuitable as a development compound. It is also pertinent that when inhalation is the intended route of delivery there are additional chemical design considerations to ensure that the physical properties required for device compatibility are met, some of which have been described in a recent special issue of this journal [72–84]. These properties – chemically stable (solid form, in solution and in the presence of acceptable excipients such as lactose); suitable crystalline form, nonhygroscopic, compatibility with micronization (thermally stable and nonabrasive) do not arise effortlessly from first generation leads elaborated from substrate library screens. For peptidic compounds destined for delivery by inhalation, a biological property that must be managed in chemical design is susceptibility to peptidases. This can be avoided by capping the molecule to confer resistance to exopeptidases and using unnatural amino acids or generating cyclic peptides to improve stability to endo- and exopeptidases.

Compared with other proteolytic enzymes, less success had been achieved until recently in the design of drug-like cysteine peptidase inhibitors. Functional groups behaving as Michael acceptors and acyloxymethyl ketones are known to act as inhibitors of cysteine peptidases and have been investigated for drug discovery. Billson *et al.* explored a series of potential inhibitors based around a potent peptide-targeting motif for Der p 1 [85]. Aldehyde **3** (FIGURE 2) was found to be a potent inhibitor of Der p 1 but, surprisingly, vinylogous esters and sulphones elaborated from it were not, despite being potent and selective inhibitors of cathepsins O2, L and S [86,87]. However, potent and relatively selective acyloxymethyl ketones based around the scaffold Val-Ala-Nle-Ser, and exemplified by **4**, **5**, **6** were identified (FIGURE 3) [85]. Based on docking in the active site of the crystal structure of Der p 1 (PDB: A2S8), the Nle carbonyl in these compounds forms a hydrophobic contact with the P₁ pocket involving a covalent bond with Cys114 in Der p 1 and a potential hydrogen bond with the carbonyl of Tyr249. The Ala carbonyl and amino functions hydrogen bond with Asp154 and the preference for small groups at P2 arises because of Ile156. The valine side chain in the substrate forms a hydrophobic contact with the P₃ pocket and the end capping groups form hydrophobic associations, including possible π -stacking interactions with Tyr residues in the enzyme. Because they are very reactive, inhibit through an irreversible covalent mechanism potentially capable of generating haptens and lack selectivity, compounds of this type were deemed undevelopable, especially in this therapeutic indication where the intended patient population has a genetic predisposition to allergy.

We have evaluated several series of compounds during the design of reversible inhibitors of Group 1 HDM allergens. Some, such as the acyloxymethyl ketone ADZ 50,000, have been used as tool compounds to explore details of Der p 1 enzymology and to probe the biological effects of Der p 1 in experimental models [33,88]. Based on the ability of early inhibitors, including ADZ 50,000, to inhibit the allergen-dependent increase in airway permeability, transepithelial allergen delivery and allergic sensitization [42,88], we refer to this new class of drugs as ‘**allergen delivery inhibitors**’ (ADIs). Using the substrate preferences of Der p 1 as a design template we have focussed efforts on optimizing the potency and biological stability of potential inhibitors. Initial efforts were focused on amino ketones

exemplified by compounds **7–12** (FIGURE 4). Compound **8** (ADZ 51,111) proved a useful scaffold for rapid exploration of SAR and fine tuning of physical properties. The presence of a basic centre provided options for salt formation to aid the manipulation of physical properties and with an AlogP of 2.9, it was reasonable to infer that these compounds would have acceptable lung retention. D-amino acids were found to be tolerated at the P₃, although not the P₂, position and provided one means of improving stability, whilst optimization of the capping groups at each end of the peptide scaffold was investigated for potency enhancement and providing resistance to exopeptidases. Docking studies with the A2S8 crystal form of Der p 1 allowed us to compare the putative binding interactions of the irreversible covalent inhibitor **5** with those of the reversible covalent inhibitor ADZ 51,111, providing a design rationale from which compounds with improved properties could be elaborated (FIGURE 5). In addition, investigation of alternative warheads, such as the aldehyde **13** identified high potency inhibitors that were optimized for selectivity.

Recently, data have been disclosed that show that another compound, ADZ 51,457, is a potent and enduring inhibitor of the recruitment of eosinophils and DCs to the airways of sensitized rats and mice challenged with a mixture of the

Key Term

Allergen delivery

inhibitor: New class of drugs directed at asthma triggers exemplified by inhibitors of Group 1 cysteine peptidase house dust mite allergens.

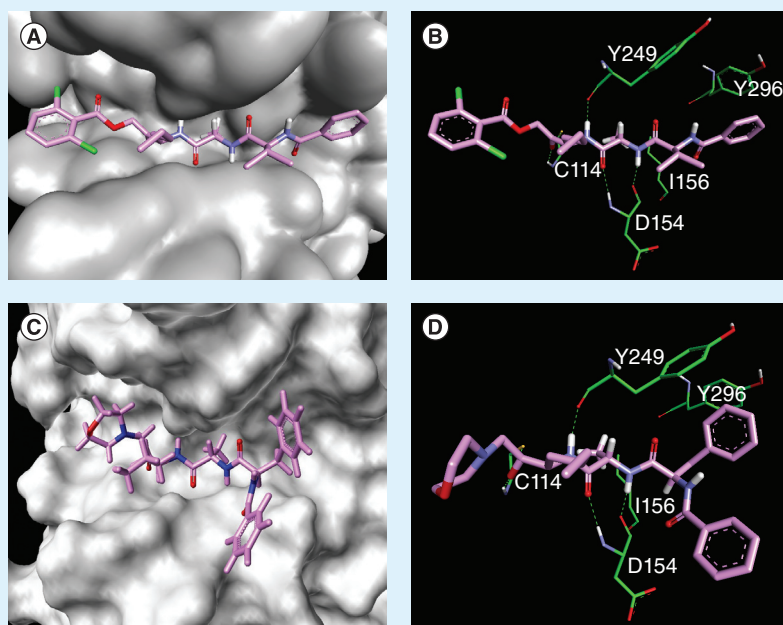


Figure 5. Docking of compound 5 in the catalytic cleft of Der p 1 (PDB code: A2S8). (A) Surface representation, (B) a simplified 3D view showing key hydrogen bonding interactions between inhibitor and target. (C & D) A corresponding image pair for ADZ 51,111.

full gamut of HDM allergens [89,90]. The reduction in cell recruitment occurs in parallel with an inhibition of chemokine release [90]. An informative feature of these findings is the appreciable inhibition of the HDM challenge that can be obtained by a peptidase inhibitor targeting only the Group 1 allergen component of the mixture. This suggests that IgE-independent actions of the Group 1 peptidases, such as Der p 1, have a key role in the responses to HDM allergens.

These emerging data provide encouraging evidence that a small-molecule intervention directed the major trigger of allergic asthma can attenuate signaling through pathways that are centrally involved in the pathogenesis of the disease. Targeting a single HDM allergen from an array of other potentially immunogenic proteins appears to be efficacious because of exploitable inequalities in allergen importance that may have no simple relationship to the intrinsic immunogenicity of a protein. There are numerous potential advantages to intervention at such a high level in the allergy cascade, notably the ability to inhibit responses to specific triggers without the pan-immunosuppressant effects of ICS. This is likely to be of significant

benefit. Drug design advantages accrue from the target being nonhuman and the inhibitor-target interaction being entirely extracellular, which enable freedom in chemical design. There is clearly much to be done to validate the ADI approach, but drugs are now emerging that will enable this new therapeutic concept to be properly evaluated in preclinical and clinical models.

Financial & competing interests disclosure

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Executive summary

Background

- Historically, it has been assumed that new medicines for asthma will continue to be found by identifying more targets among the complex effector pathways in disease.

Need for a novel approach to asthma therapy

- Overwhelming evidence shows this approach to be flawed because complex networks have evolved to possess resilience: the greater the number of targets the lower the probability of producing an effective medicine.
- Network resilience means that downstream interventions need to have a broad spectrum of effects, like ICS, but this is achieved at the expense of a wider range of potential adverse events.

Allergen delivery inhibitors

- Advances in immunological understanding have created new drug discovery opportunities for allergic asthma, targeting the triggers of the disease rather than its effector mechanisms.
- Although studies are in their infancy, encouraging data have been obtained for Allergen Delivery Inhibitors, a new group of drugs that inhibit the cysteine peptidase activity of Group 1 mite allergens.
- Allergen Delivery Inhibitors have been shown to inhibit the recruitment of eosinophils and prevent the recruitment and activation of dendritic antigen presenting cells that are central to the initiation and maintenance of allergic sensitization.

Potential advantages

- Targeting a nonhuman trigger of asthma brings potential advantages. Because the target is only present in the airway lumen, there is no requirement for a systemic component to drug action that is advantageous from a safety perspective and is ideally suited to inhalation as a method of delivery. Furthermore, because target-inhibitor engagement is extracellular there are no requirements to design molecules that are cell permeant.

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