

The Discovery of Potent, Selective, and Reversible Inhibitors of the House Dust Mite Peptidase Allergen Der p 1: An Innovative Approach to the Treatment of Allergic Asthma

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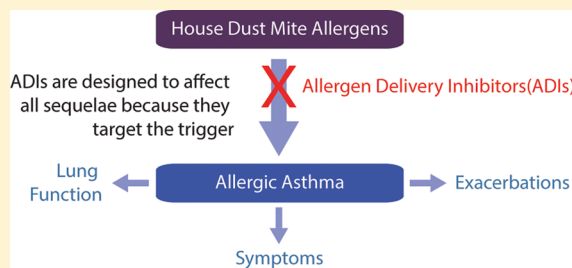
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S Supporting Information

ABSTRACT: Blocking the bioactivity of allergens is conceptually attractive as a small-molecule therapy for allergic diseases but has not been attempted previously. Group 1 allergens of house dust mites (HDM) are meaningful targets in this quest because they are globally prevalent and clinically important triggers of allergic asthma. Group 1 HDM allergens are cysteine peptidases whose proteolytic activity triggers essential steps in the allergy cascade. Using the HDM allergen Der p 1 as an archetype for structure-based drug discovery, we have identified a series of novel, reversible inhibitors. Potency and selectivity were manipulated by optimizing drug interactions with enzyme binding pockets, while variation of terminal groups conferred the physicochemical and pharmacokinetic attributes required for inhaled delivery. Studies in animals challenged with the gamut of HDM allergens showed an attenuation of allergic responses by targeting just a single component, namely, Der p 1. Our findings suggest that these inhibitors may be used as novel therapies for allergic asthma.



INTRODUCTION

At the heart of the clinical management of asthma lies a paradox: despite efficacious and safe therapies (e.g., β_2 agonist bronchodilators, inhaled corticosteroids, antileukotrienes, and an anti-IgE monoclonal antibody), the condition remains poorly controlled and its prevalence continues to increase.^{1,2} The multiple factors underlying this paradox highlight a significant unmet need which might be better addressed by a completely different approach. Current standard of care in asthma is directed at downstream effector mechanisms, and the medicines involved act primarily by relieving symptoms. For novel therapies in development, target selection has been primarily driven by a focus on deeper understanding of the effector pathways of asthma, with the hope that new nonsteroidal interventions will reduce both the risk of disease exacerbations, which is the major goal of clinical management, and the potential for adverse events. However, experience shows that only limited success has been achieved by targeting individual downstream effectors in asthma, highlighting the need for new approaches.

For any condition, an alternative to symptom management is to target the major trigger or root cause. However, asthma is a complex spectrum of conditions rather than a homogeneous

disease and on first inspection such an approach seems unfeasible. Asthma may be broadly divided into nonallergic and allergic types, with the latter, triggered by inhaled environmental allergens, predominating. Two pieces of epidemiological evidence suggest that design of an intervention directed toward a trigger of allergic asthma could be surprisingly tractable. First, a succession of studies highlight that, globally, the most important providers of allergen triggers are house dust mites (HDM).^{3–12} Second, sensitization to HDM precedes the development of sensitization to allergens from unrelated sources.^{13,14} Mechanistically, this longitudinal relationship exists because HDM facilitate sensitization to other agents by providing essential collateral priming events on which other allergens depend.

HDM are sources of more than 20 denominated allergen groups,¹⁵ with those of group 1 being of particular interest because of their abundance, allergenicity, and their functional properties which promote sensitization to themselves and other allergens.^{15–24} Sensitization to HDM allergens occurs through inhalation of this animal's fecal pellets, which, when they impact

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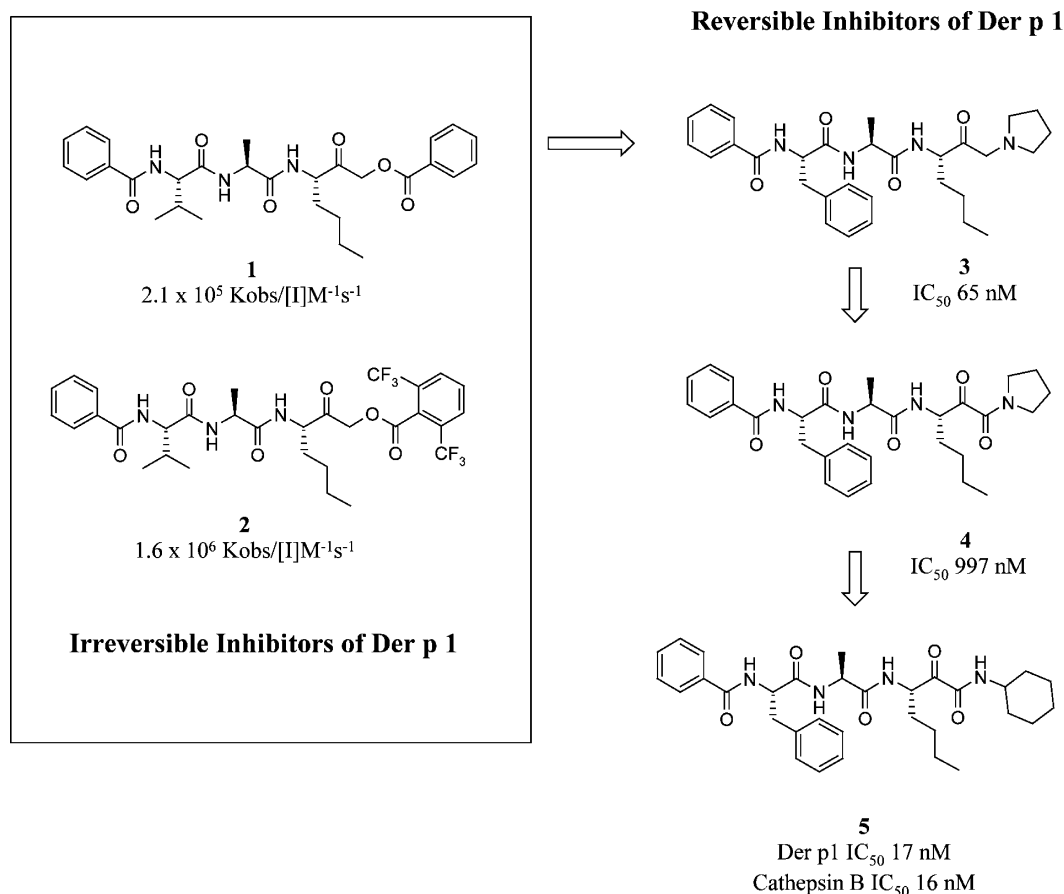


Figure 1. Reversible Der p 1 inhibitors were derived from a series of irreversible inhibitors by modifying the cysteine binding motif. This led to the identification of pyruvamide inhibitor 5.

upon the airway mucosa, hydrate and release their contents. The group 1 allergens (e.g., Der p 1, Der f 1, Eur m 1) of the various HDM species form a distinct subfamily of C1 cysteine peptidases²⁵ whose sequences are sufficiently identical that targeting them with a single agent is a realistic possibility.²² Two general peptidase-dependent mechanisms have been identified by which group 1 HDM allergens promote allergic sensitization and asthma. The first is their ability to cleave epithelial tight junctions by proteolytic attack on the transmembrane adhesion domains of occludin and claudin family proteins.^{24,26} This cleavage results in the epithelial barrier becoming leaky, increasing the probability of contact of any allergen with dendritic antigen-presenting cells and permitting the migration of these cells, along with secondary effector cells, into the airway lumen.^{6,27,28} Their second general mechanism as proteases is to activate signal transduction pathways of innate immunity which release chemokines and other mediators (e.g., IL-13, IL-33, TSLP, IL-25, CCL-20)^{16,18,29} that are known to recruit the necessary effector cells and promote a TH₂ bias to immune responses.^{6,18,23,29–31} Significantly, evidence suggests that some of these innate immune mechanisms are the focus of important genetic predispositions for allergic asthma.³²

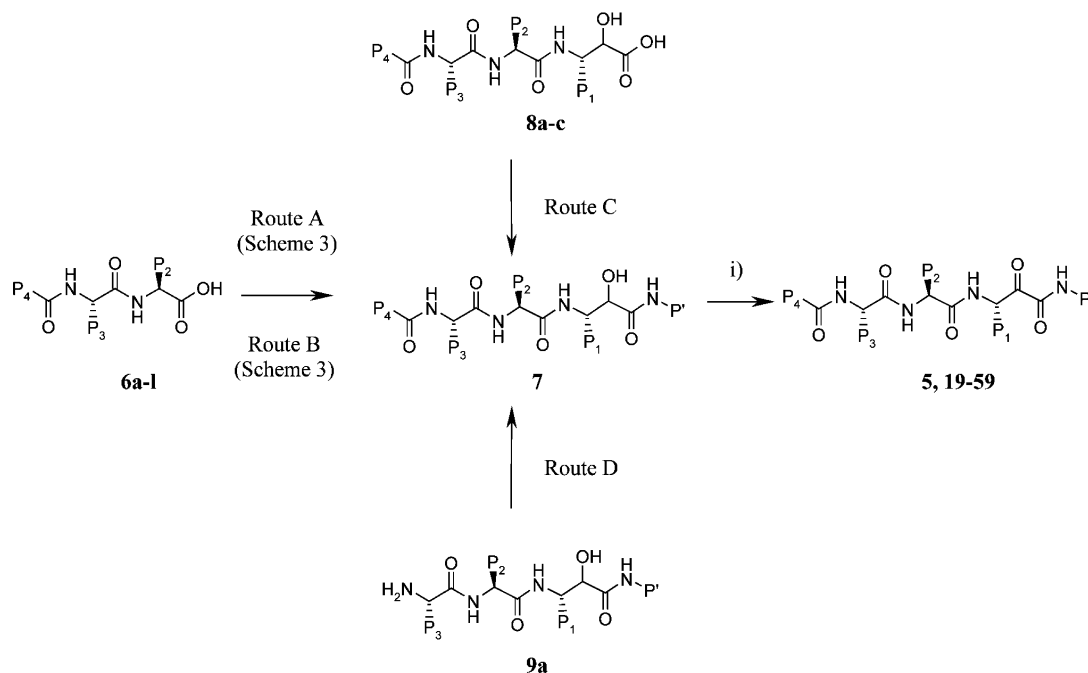
Given the importance of HDM sensitization as a trigger for asthma and the increasing recognition that the peptidase activity of group 1 HDM allergens plays an important role in both its initiation and maintenance, the aim of our program was to develop small-molecule inhibitors of these pivotal allergens. We call these new drugs “allergen delivery inhibitors” (ADIs), and it is our hypothesis that an ADI compound would provide

an effective inhaled treatment for patients suffering from allergic asthma. The compounds disclosed herein are the subject of a patent disclosure.³³

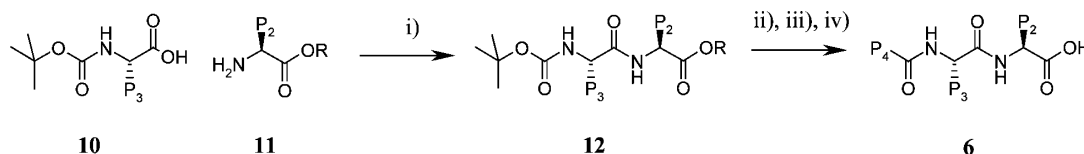
RESULTS AND DISCUSSION

Identification of Reversible Der p 1 Inhibitors. Prior to the commencement of our program, the only reported inhibitors of Der p 1 were irreversible acyloxymethyl ketone inhibitors (Figure 1).³⁴ Given that asthma treatment will require chronic drug administration, we considered that compounds having an irreversible mechanism of action were inherently lacking in developability because of their potential to elicit adverse effects. We therefore sought to replace the irreversible binding motif with functional groups that could form a fully reversible covalent bond with the active site cysteine residue. An investigation of amino-ketones afforded compounds with initially encouraging potency against Der p 1, but we were unable to optimize inhibitory activity beyond that shown by compound 3. To enhance binding to the active site cysteine residue, we therefore examined alternative groups, including the corresponding pyruvamide analogues. This rapidly led to the identification of compound 5, which became the starting point for our discovery program (Figure 1).

Low molecular weight peptides are often characterized by poor oral bioavailability. This has been attributed to their propensity for proteolytic cleavage, and the presence of a high proportion of hydrogen bond donors and acceptors, in conjunction with a relatively flexible scaffold; factors likely to hinder passive absorption in the gut. Conversely, these

Scheme 1. General Route to Pyruvamide Inhibitors of Der p 1^a

^a(i) Dess–Martin periodinane, DCM.

Scheme 2. General Procedure for the Solution-Phase Synthesis of Dipeptide Acids^a

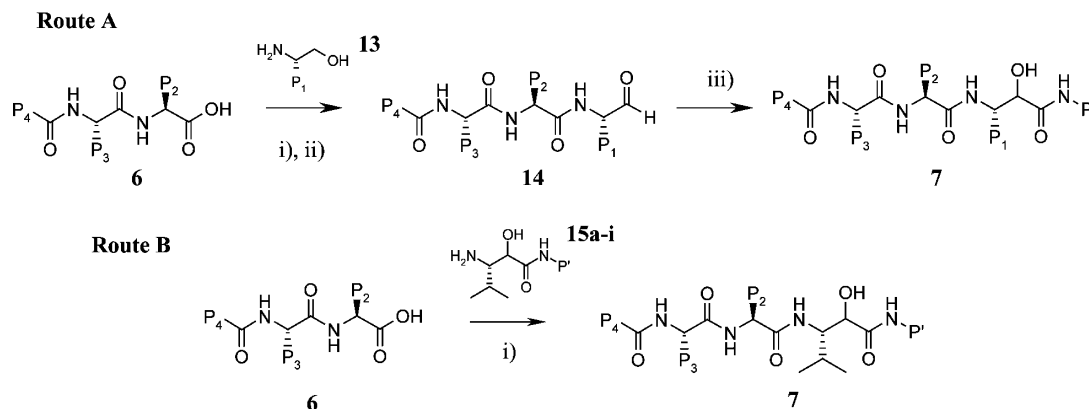
^a(i) *i*BuOCOCl, *N*-methylmorpholine, $-40\text{ }^{\circ}\text{C}$, THF; (ii) TFA, DCM or 4 N HCl/dioxane; (iii) $\text{P}_4\text{-CO}_2\text{H}$, TBTU, DIPEA, DCM; (iv) LiOH, THF/ H_2O .

properties may be beneficial for an inhaled drug as they would minimize absorption of any inadvertently swallowed portion of a dose, thereby limiting adverse systemic effects. In our view, these considerations made a peptidic scaffold an ideal template for the design of an inhaled Der p 1 inhibitor.

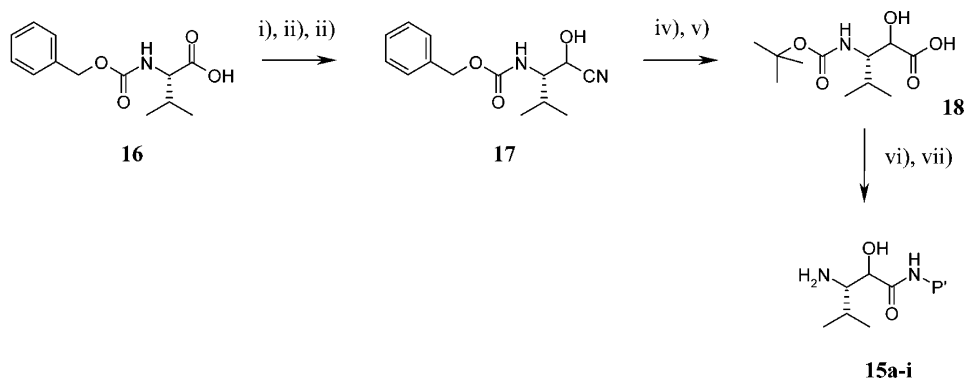
To obtain *in vivo* efficacy that was compatible with delivery from a range of inhaler devices, we required a compound with high inhibitory potency against Der p 1. Upon the basis of empirical estimates of target exposure, we therefore set a template $\text{IC}_{50} \leq 20\text{ nM}$ for the target. While compound 5 fulfilled this criterion, a developable candidate requires other features that impact on *in vivo* efficacy, notably those properties that would affect the retention of the compound at the site of action: permeability, lipophilicity, and stability in the lungs. Additionally, increasing the intrinsic selectivity of our initial lead over closely related human cysteine peptidases, notably cathepsin B (Cat B) (Figure 1), was desirable because intrinsic selectivity combined with low systemic exposure would reduce the risk of off-target events. This risk would be subject to further mitigation because of the entirely extracellular interaction between target and inhibitor, whereas the potential off-target enzymes have largely intracellular dispositions which would require compounds to be highly membrane permeant for enzymes to be at risk of inhibition. Pharmaceutical properties were also taken into account early in the program so as to generate compounds that would be suitable for use in a dry-

powder inhaler (DPI), our preferred device. The requirement to be able to consistently produce particles of optimal respirable size places stringent constraints on the solid-phase behavior of compounds that are to be delivered by DPI. For example, the need to be compatible with micronization requires a stable, nonhygroscopic crystalline form with a melting point $>100\text{ }^{\circ}\text{C}$. Aqueous solubility was required to balance adequate drug dissolution for efficacy with an enduring effect while avoiding the potential for irritancy associated with low solubility compounds. Compound 5 lacked selectivity and was extensively ($>50\%$) degraded by airway macrophages over a 2 h period, suggesting that its duration of action *in vivo* would be insufficient. However, its potency and the scope for structural variation suggested it was a promising lead for optimization.

Synthetic Chemistry. Pyruvamide-motif inhibitors were synthesized from the corresponding α -hydroxy amides (7) by oxidation with the Dess–Martin periodinane reagent (Scheme 1). The α -hydroxy amide intermediates were constructed by two synthetic approaches: either a modified Passerini reaction³⁵ (route A) or the use of cyanohydrin chemistry (route B). Both routes required capped dipeptide acids (6), produced either by solid- or solution-phase synthesis. Solid-phase synthesis was carried out using standard Fmoc chemistry on Wang resin.³⁶ In-solution chemistry used a stepwise process which involved, as a typical first step, the coupling of a P_2 amino acid ester (11) with a Boc-protected P_3 amino acid (10) under low-temperature

Scheme 3. General Procedures for the Synthesis of α -Hydroxy Amide Tripeptides^a

^a(i) *i*BuOCOCl, *N*-methylmorpholine, -40 °C, THF; (ii) Dess–Martin periodinane, DCM; (iii) P'N⁺≡C⁻, pyridine, TFA, DCM.

Scheme 4. General Procedure for Synthesis of β -Amino- α -hydroxy Amide Intermediates^a

^a(i) *N*-Methyl-*O*-methyl-hydroxyl amine, EDC, DIPEA, DCM; (ii) LAH, THF; (iii) NaHSO₃, NaCN, H₂O/THF; (iv) 6 N HCl, dioxane, 100 °C; (v) Boc₂O, Et₃N, MeOH; (vi) diphosgene, H₂NP', DCM; (vii) TFA, DCM or 6 N HCl dioxane, THF.

mixed-anhydride coupling conditions to avoid epimerization of the P₃ chiral center (Scheme 2).

For route A, the dipeptide acids (**6**) were typically coupled with the appropriate amino alcohol (**13**) followed by oxidation to give the corresponding aldehyde (**14**). A modified Passerini reaction was then used to produce the α -hydroxy amide (Scheme 3).³⁵ For route B, a variety of α -hydroxy amides (**7**) were synthesized by coupling β -amino- α -hydroxy amides (**15**) with dipeptide acids (**6**) under similar conditions to those previously described (Scheme 3). The β -amino- α -hydroxy amides (**15**) were made in seven steps from Cbz-protected valine **16** (Scheme 4).

Finally, some compounds were made by routes C and D, each being a variation on route B where the order of events was changed to aid the synthesis of particular analogues (Scheme 1).

Modeling the Binding Mode to Der p 1. To assist the design of analogues of compound **5**, a computational model was constructed based on the crystal structure of Der p 1 (PDB code 2AS8) and on the structures of a number of peptidic inhibitors bound to the C1 family of clan CA peptidases (PDB codes 1TU6 and 2BDL). Compound **5** was built and minimized within the active site of the Der p 1 crystal structure. The electrophilic carbonyl of the pyruvamide was positioned to form a covalent interaction with the catalytic cysteine residue (Cys 34), while the peptide backbone of **5** was oriented to follow a similar trajectory to that of the other

peptidic inhibitors. This minimized structure revealed a number of putative hydrogen-bonding interactions which anchored compound **5**, thereby allowing the side chains to interact with the specificity pockets of the enzyme. On the basis of this binding model, we propose that the amide carbonyl of the pyruvamide unit interacts with the backbone NH of Cys 34, the NH of the P₁ subunit is able to form an interaction with the carbonyl of Tyr 169, and the P₂ subunit forms a donor–acceptor pair with the backbone carbonyl and NH of Asp 74 (Figure 2a).

Improving Selectivity. Significant off-target activity of compound **5** against Cat B was revealed by counter-screening against human cysteine peptidases, notably certain members of the C1 family. We therefore investigated SAR around compound **5** with the aim of improving the selectivity profile (Table 1). We established that the P' cyclohexyl group could be replaced with a benzyl group without a significant effect on Der p 1 potency or selectivity (for comparison see **19** and **20**; similar effects seen for other analogues, data not shown), so these two groups were used interchangeably for SAR comparisons. As the pyruvamide motif has the potential to interact indiscriminately with nucleophilic cysteine or serine residues of other peptidases, we hoped that increasing the steric bulk of the P₁ substituent would hinder nonspecific interactions. Increasing the branching at P₁ to *iso*-propyl (**19**) retained inhibitory potency on Der p 1 but did not significantly improve selectivity over Cat B. It did however improve

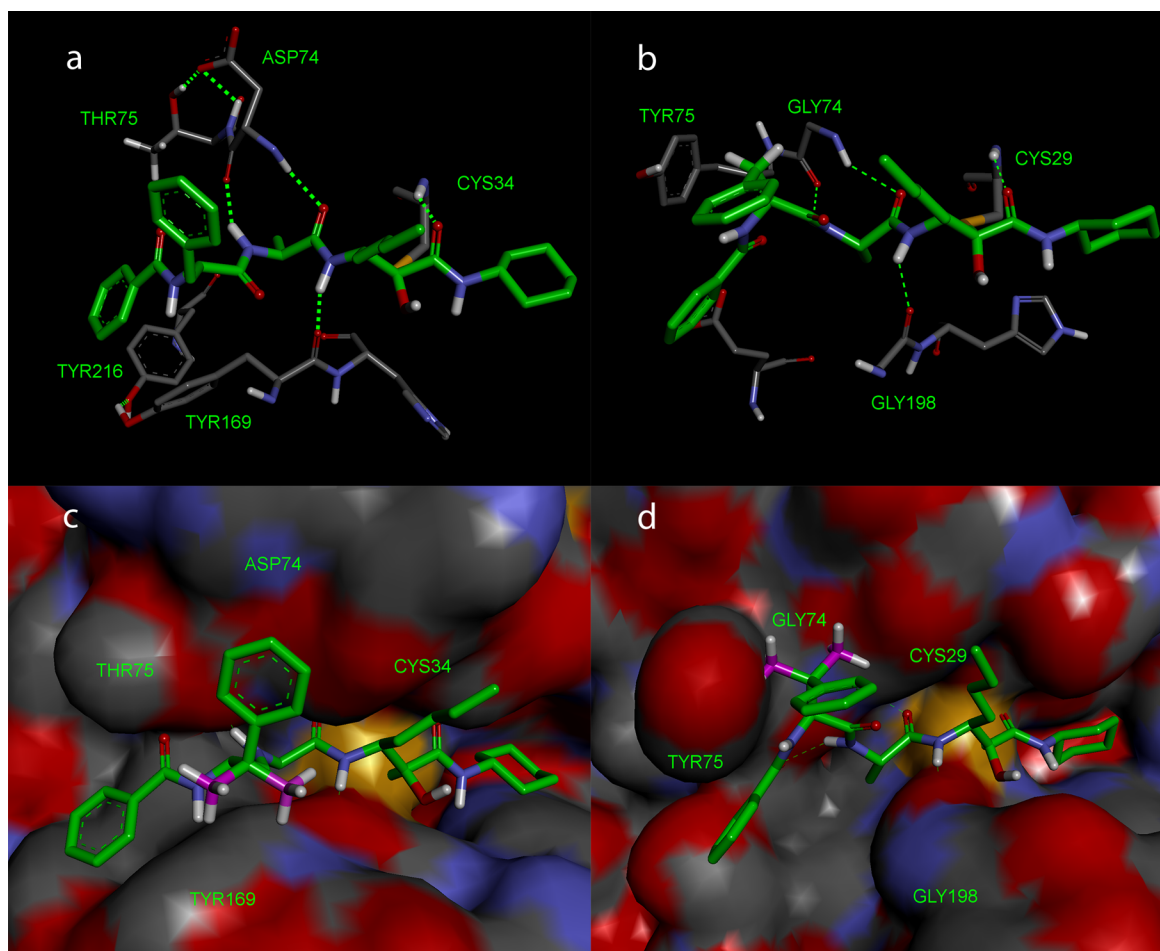


Figure 2. Model of compound **5** bound to Der p 1 (PDB: 2A58) and Cat B (PDB: 1GMY). (a) Proposed binding mode of compound **5** (green) to Der p 1 active site. (b) Proposed binding mode of compound **5** to Cat B active site. (c,d) Proposed incorporation of dimethyl group (pink) as part of the P₃ substituent bound to Der p 1 and Cat B respectively. The bulky P₃ residue is anticipated to clash with Tyr 75 in Cat B.

Table 1. Impact of Modifying P₁, P₂, and P₃ Groups on Der p 1 Potency and Selectivity over Cat B

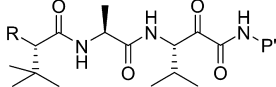
| compd no. | P ₁ | P ₂ | P ₃ | P' | Der p 1 IC ₅₀ (nM) | Cat B IC ₅₀ (nM) |
|-----------------------|----------------|----------------|-----------------------|------------|-------------------------------|-----------------------------|
| 5 | <i>n</i> -Bu | Me | benzyl | cyclohexyl | 8 ± 1 | 17 ± 2 |
| 19^a | <i>i</i> -Pr | Me | benzyl | cyclohexyl | 18 ± 2 | 52 ± 5 |
| 20 | <i>i</i> -Pr | Me | benzyl | benzyl | 12 ± 2 | 50 ± 5 |
| 21 | <i>t</i> -Bu | Me | benzyl | cyclohexyl | 9167 ± 880 | ND ^b |
| 22 | <i>i</i> -Pr | Me | <i>t</i> -Bu | cyclohexyl | 14 ± 3 | 378 ± 27 |
| 23 | <i>i</i> -Pr | Me | C(Me) ₂ Ph | benzyl | 42 ± 6 | 446 ± 11 |
| 24 | <i>i</i> -Pr | <i>n</i> -Pr | benzyl | cyclohexyl | 164 ± 24 | 67 ± 1 |


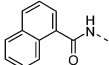
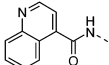
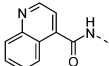

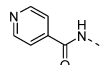
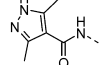
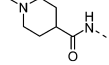
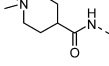
^aContains ~30% of P₁ R epimer. ^bND = not determined.

resistance to processing by airway macrophages, with ~70% remaining unchanged after 2 h. The *iso*-propyl group was retained for further investigations because further branching to *tert*-butyl (**21**) reduced the potency against Der p 1. Of further note, removal of the P₁ substituent resulted in a 10–20-fold drop in Der p 1 potency (see Supporting Information, Appendix 5, compound **76**).

Comparison of the reported crystal structures of Der p 1 and Cat B revealed that the S₃ pocket is more capacious in Der p 1 than in Cat B, mainly due to the presence of a Thr in Der p 1

(Thr 74) instead of Tyr in Cat B (Tyr 75). Cathepsins S (Cat S) and K (Cat K) also possess similar large groups at this position (Phe 70 in the case of Cat S and Tyr 67 in the case of Cat K). We hypothesized that increasing the steric bulk at P₃ would increase selectivity with respect to these enzymes (Figure 2c,d), and it was therefore pleasing to find that switching from benzyl (**5**) to *tert*-butyl (**22**) increased selectivity over Cat B while maintaining good inhibitory potency on Der p 1. Similarly, introducing geminal-dimethyl substitution onto the benzyl group (**23**) reduced inhibitory

Table 2. Impact of Modifying the P₄ Substituent on log *D*, Der p 1 Potency, and Selectivity over Cat B


| Cpd # | R | P' | Der p 1 IC ₅₀ (nM) | Cat B IC ₅₀ (nM) | Log <i>D</i> _{7,4} ^c |
|-----------------|---|------------|-------------------------------|-----------------------------|--|
| 22 |  | cyclohexyl | 14 ± 3 | 378 ± 27 | 3.9 |
| 25 |  | cyclohexyl | 20 ± 3 | 721 ± 35 | 4.3 |
| 26 |  | cyclohexyl | 13 ± 2 | 628 ± 47 | 3.5 |
| 27 |  | benzyl | 18 ± 1 | 3404 ± 187 | 3.4 |
| 28 |  | benzyl | 7 ± 1 | 429 ± 65 | 3.6 |
| 29 |  | cyclohexyl | 13 ± 1 | 231 ± 17 | 2.8 |
| 30 ^a |  | benzyl | 67 ± 10 | ND ^b | ND ^b |
| 31 |  | benzyl | 19 ± 2 | 283 ± 50 | ND ^b |
| 32 |  | benzyl | 6 ± 1 | 274 ± 44 | -0.9 |

^aContains ~30% of P₁ R epimer. ^bND = not determined. ^cSingle determination measured as described in Supporting Information.

potency against Cat B with only a modest impact on Der p 1 activity. Compound **22** also showed resistance to airway macrophages, with no significant degradation observed over 2 h.


Further modeling suggested that the S₂ pocket was shallow in Der p 1 compared to cathepsins B, K, or S; effective inhibitors of these enzymes tend to have groups larger than methyl at this position.^{37–39} Consistent with these observations, the methyl substituent at P₂ generally provided the best balance of potency and selectivity for Der p 1 (**19** vs **24**).

Modification of Molecular and Physicochemical Properties. Preliminary investigations into the P₁, P₂, and P₃ positions had shown that we could obtain good inhibitory potency against Der p 1 and significantly improve the selectivity over Cat B. We next turned to the N and C terminal groups with the aim of modifying physicochemical properties to optimize the endurance and pharmaceutical properties of inhibitors. Simultaneously, we hoped to take advantage of any preferences shown in the S₄ and S' pockets to further enhance Der p 1 potency and selectivity with respect to Cat B.

A number of chemical design philosophies have been adopted to enhance the duration of action of small-molecule drugs aimed at other respiratory targets.^{40,41} Approaches considered potentially applicable to ADIs involved the incorporation of features to confer either low permeability or increased binding to lung tissue. Permeability can be reduced by increasing molecular weight and/or PSA, whereas lung tissue retention may be enhanced by combining increasing lipophilicity with a basic or quaternary ammonium group. However, because it was uncertain how effective or well-tolerated any of these tactics would be in the case of Der p 1 inhibitors, we decided to modify their molecular and physical properties as widely as possible, thereby maximizing the scope to manipulate their in vivo and pharmaceutical behavior.

A variety of groups were investigated in the P₄ position. Additional small lipophilic substituents on the phenyl ring were tolerated (data not shown), as were fused rings which could be used to increase molecular weight and log *D*. Furthermore, some of these groups gave improved selectivity over Cat B (compounds **25**, **26**, and **27**, Table 2). Replacement of the

Table 3. Impact of Modifying P' Substituent on log D, Der p 1 Potency, and Selectivity over Cat B



| 22 and 33-52 | | | 53 | | |
|-----------------|---|----------------|-------------------------------|-----------------------------|-----------------------------------|
| Cpd # | P' | P ₃ | Der p 1 IC ₅₀ (nM) | Cat B IC ₅₀ (nM) | Log D _{7.4} ^c |
| 22 | | <i>t</i> -Bu | 14 ± 3 | 378 ± 27 | 3.9 |
| 33 ^a | | benzyl | 55 ± 4 | 311 ± 6 | ND ^b |
| 34 | | benzyl | 115 ± 14 | ND ^b | ND ^b |
| 35 | cyclopropyl | <i>t</i> -Bu | 17 ± 1 | 417 ± 25 | 2.4 |
| 36 | H | <i>t</i> -Bu | 6 ± 2 | 20 ± 3 | 1.7 |
| 37 | Me | <i>t</i> -Bu | 24 ± 5 | 494 ± 20 | ND ^b |
| 38 | CH ₂ Ph | <i>t</i> -Bu | 9 ± 1 | 512 ± 59 | 3.2 |
| 39 | | <i>t</i> -Bu | 6 ± 1 | 984 ± 164 | ND ^b |
| 40 | | <i>t</i> -Bu | 47 ± 7 | ND ^b | ND ^b |
| 41 | CH ₂ Ph -4-(CH ₂ NMe ₂) | <i>t</i> -Bu | 8 ± 1 | 242 ± 31 | ND ^b |
| 42 | (<i>R</i>)-CH(Me)Ph | <i>t</i> -Bu | 17 ± 2 | 459 ± 28 | ND ^b |
| 43 | (<i>S</i>)-CH(Me)Ph | <i>t</i> -Bu | 117 ± 34 | ND ^b | ND ^b |
| 44 | | <i>t</i> -Bu | 11 ± 2 | 367 ± 34 | ND |
| 45 | | benzyl | 14 ± 1 | 544 ± 40 | 1.3 |
| 46 | | benzyl | 14 ± 3 | 81 ± 6 | 2.6 |
| 47 | | benzyl | 9 ± 1 | 88 ± 10 | 1.7 |
| 48 | | <i>t</i> -Bu | 14 ± 2 | >2500 | 1.0 |
| 49 | | benzyl | 4 ± 1 | >2500 | 1.6 |
| 50 | | benzyl | 10 ± 1 | >2500 | ND ^b |
| 51 | | <i>t</i> -Bu | 6 ± 0 | 511 ± 88 | 3.5 |
| 52 | | benzyl | 17 ± 2 | >2500 | -0.6 |
| 53 | | benzyl | 20 ± 3 | 540 ± 28 | 2.0 |

^aContains ~30% of P₁ R epimer. ^bND = not determined. ^cSingle determination measured as described in Supporting Information.

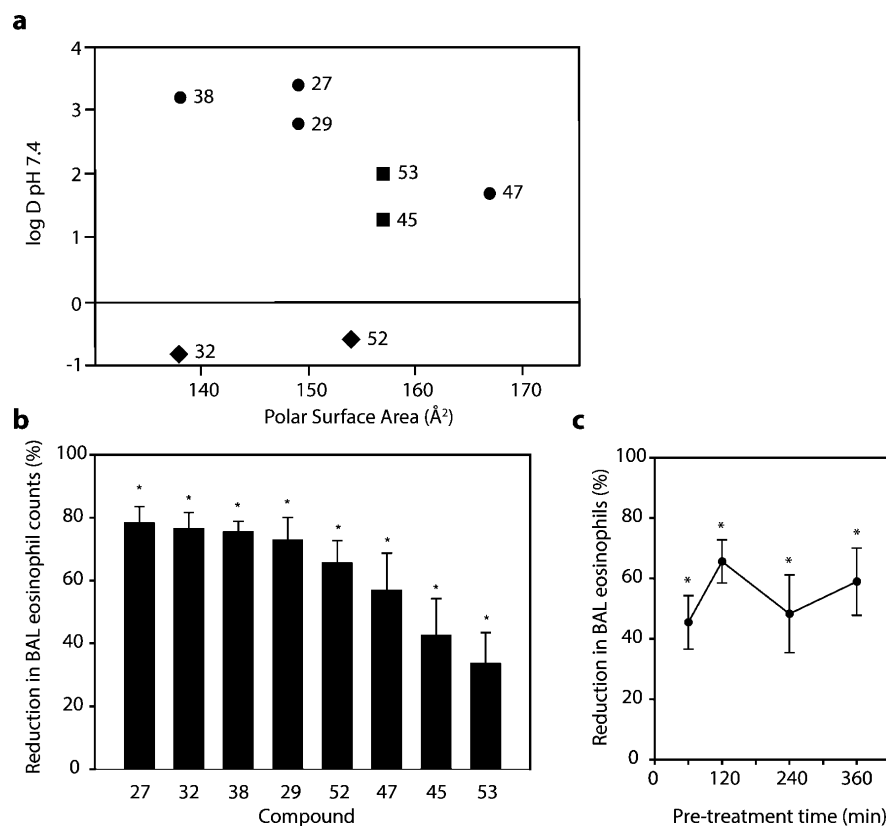


Figure 3. (a) Plot of PSA (Å²) versus measured log $D_{7.4}$ for compounds used to explore the effects of physicochemical properties on in vivo efficacy. Symbols denote the anticipated ionization state at pH 7.4: circle (neutral), square (positively charged basic center), and diamond (positively charged, quaternary ammonium). (b) Percentage reduction in the number of eosinophils recovered by BAL 48 h after challenge of nonsensitized rats with a natural mixture of HDM allergens following a single intratracheal dose of test compound 2 h prior to challenge. Compounds were dosed at a drug:target molar ratio of 15:1 (compound 38) or 50:1 (other compounds). (c) Compound 52 exemplifies the endurance of protection (>6 h) by quaternary amines. Data are shown as the percentage reduction in BAL eosinophil numbers following HDM allergen challenge after administration of a single intratracheal dose of 52 (67 nmol/kg) administered at the stated times prior to HDM allergen challenge. In (b) and (c), data are mean values \pm SE, with 10 animals per treatment group. * $P < 0.001$ (1-way ANOVA) compared to controls which were not treated with 52.

phenyl group with 5- or 6-membered heterocycles reduced log D but, with the exception of the 4-pyridyl group (29), these decreased the potency against Der p 1. It was also possible to incorporate a basic center in the form of an *N*-methylpiperidine group (31) which could be quaternized to give 32, which as expected showed excellent aqueous solubility (~ 1.6 mmol when shaken in PBS_{7.4} for 2 h). The S_4 pocket in Der p 1 is surrounded by a number of Tyr residues (Tyr 169, Tyr 216, Tyr 218), and we speculate that the positively polarized *N*-methyl hydrogen atoms of 32 are able to make favorable interactions with these residues, thereby resulting in a potent Der p 1 inhibitor.

Having shown that cyclohexyl and benzyl gave good activity in the P' position, we next turned to exploration of the SAR in this region. Because the parent compounds, such as 22, were lipophilic, we initially focused on reducing log D because this would increase aqueous solubility and mitigate any potential risk of irritancy arising from the accumulation of poorly soluble compounds in the lung. Replacement of the C-4 cyclohexyl carbon with a heteroatom lowered both Der p 1 and Cat B inhibitory activity and was not pursued further (Table 3, 33 and 34). It transpired that a more successful means of lowering log D was to reduce the ring size. Replacement of the cyclohexyl group (22) with a cyclopropyl group (35) reduced the measured log D by ~ 1.5 log units while maintaining high potency against Der p 1 and selectivity over Cat B, whereas

removal of the P' group caused attrition of selectivity even though potency was acceptable (36). However, simple substitution with a methyl group was sufficient to restore selectivity (37). Regardless of these findings, we discovered that the synthesis of P' benzyl analogues was a more fruitful approach to the modification of physicochemical properties and the optimization of both potency and selectivity. In general, lipophilic groups in the *para* position were less active than more polar groups, especially those containing a hydrogen bond donor. In particular the introduction of a *p*-SONH₂ group, as in compound 39, gave high Der p 1 potency and excellent selectivity over Cat B. Introduction of an α -methyl group showed a preference for the R enantiomer (42). Basic groups could also be appended to the phenyl ring as shown by compound 41.

To further probe the SAR of the P' pocket, we elected to explore a series of glycinamide analogues, reasoning that the glycinamide moiety could mimic the peptidic backbone of a substrate molecule and that the amide substituent would allow us to modify the molecular and physical properties. Furthermore, this subseries would have an increased PSA, thereby reducing compound permeability in both the lung and the gut. Serendipitously, this was an effective way of removing the undesired Cat B activity. Compound 45, containing a basic motif, showed excellent Der p 1 inhibitory activity and good selectivity over Cat B. Moreover, combining this modification

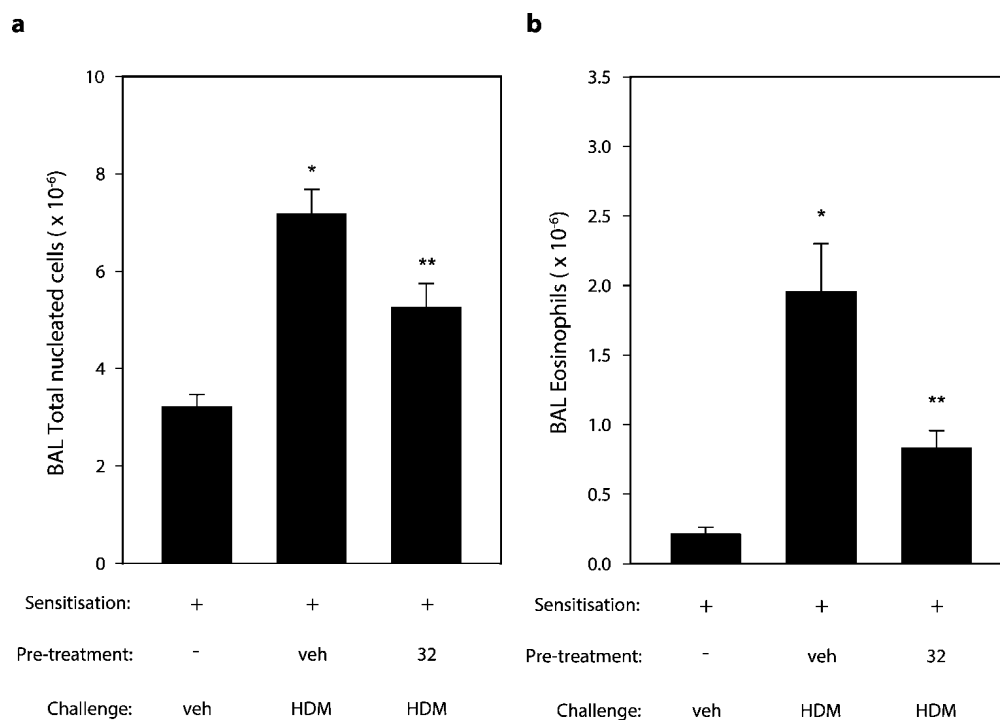


Figure 4. Effect of single dose pretreatment with compound 32 (12 $\mu\text{g}/\text{kg}$; 18 nmol/kg) on the recruitment of cells to the airways 48 h following challenge with mixed HDM allergens in sensitized BN rats. The compound was administered 2 h prior to HDM allergen challenge. (a) Data for total nucleated cells recovered by BAL; (b) corresponding data for eosinophils. Results are shown as mean \pm SE. Treatment groups comprised 10 animals. * $P < 0.01$ versus vehicle (veh)-challenged animals sensitized to HDM. ** $P < 0.001$ versus animals pretreated with vehicle and then challenged with mixed HDM allergens (1-way ANOVA).

with a *tert*-butyl group in P_3 resulted in compound 48 that was both potent against Der p 1 and which showed no significant inhibition of Cat B at 2.5 μM . Alternatively, replacing the *N*-methyl group with a bulkier *iso*-propyl group (49) or introducing a substituent on the α position of the glycinamide (50), reduced inhibition of Cat B without the need to introduce the *tert*-butyl group at P_3 . As can be seen from Table 3, glycinamides that were potent against Der p 1 spanned a range of lipophilicity. Additionally, it was possible to convert the piperazine group to a quaternary ammonium compound (52) with good Der p 1 activity and excellent selectivity over Cat B.

To further examine the selectivity of inhibitors produced by P' variation, compounds were counter-screened against a wider panel of proteases and it was pleasing to see that good selectivity was attained over a diverse range of targets. As general exemplification of a compound which displays encouraging potency against the Group 1 HDM peptidase allergens, data for compound 38 are presented in Appendix 7 of the Supporting Information.

Pharmaceutical Properties. A key goal of the program was to produce potential candidate drugs with stable crystalline forms compatible with delivery by DPI and with confidence that they would be compatible with other devices. Several approaches were taken to increase the likelihood of obtaining crystalline compounds. However, it was difficult to predict with confidence which would crystallize; after establishing a viable approach, approximately half of the compounds examined crystallized readily, and it is probable that others could be crystallized with further effort. Tactics used to favor crystallinity are described in the Supporting Information. The fact that satisfactory crystalline properties were common and associated with molecules whose profiles could be manipulated by

structural alterations at multiple locations meant that we were able to prosecute the program with confidence that all of the required characteristics for a candidate drug were achievable within this series.

Efficacy in Vivo. Having demonstrated that it was possible to obtain potent, selective inhibitors of Der p 1 spanning a range of physicochemical properties and that no overriding issues with crystallinity existed, experiments were carried out to elucidate which features were most strongly correlated with in vivo efficacy. All of the compounds chosen for this work showed no degradation when incubated with rat airway epithelial cells or airway macrophages for 2 h, thereby minimizing metabolic instability as a variable in these studies.

A series of similarly potent compounds with a range of measured $\log D_{7.4}$ values and PSA values (Figure 3a) were chosen for study in rats challenged with a natural mixture of allergens from *Dermatophagoides pteronyssinus*, i.e., a diverse mixture containing the full spectrum of HDM allergens including our drug target, Der p 1. We had previously discovered that when delivered by intratracheal aerosol to rats, the proteolytic activity of Der p 1 in this mixture recruited inflammatory cells to the airways *in the absence of prior sensitization* because the proteolytic activity triggered innate immune responses. The nature and time-course of this cell recruitment was similar to that which occurs in animals sensitized to HDM but was of smaller magnitude. As a first step, we therefore exploited this finding of a peptidase-dependent innate response to compare the activity of selected Der p 1 inhibitors. In brief, this model involved intratracheal aerosolization of a solution of the test compound into rats using a Penn-Century device, followed 2 h later by challenge with a mixture of HDM allergens delivered by the same method.

Bronchoalveolar lavage (BAL) was performed 48 h after allergen challenge as the optimal time to evaluate any effects on the recruitment of eosinophils (data not shown). The compounds under investigation contained a range of groups that would be expected to be neutral or positively charged at physiological pH. The percentage inhibition of eosinophil recruitment when challenged with the HDM allergens was recorded (Figure 3b). There was a clear trend toward more lipophilic compounds showing better efficacy (compare compounds 27, 29, and 38 to compounds 45, 47, and 53). Good efficacy was also achieved for the quaternary ammonium compounds 32 and 52 with good endurance of action (>6 h protection from a single dose) evident for both (Figure 3c). Further details of the duration of protection achieved with a selection of inhibitors are presented in Appendix 8 of the Supporting Information.

Demonstration of potency and endurance of action against the HDM allergen target in the innate response model led us to investigate efficacy in an IgE-dependent context in animals actively sensitized to a mixture of HDM allergens. Figure 4a shows that a single dose of compound 32 administered 2 h prior to allergen challenge significantly blunted the increase in total nucleated cells recoverable from the airways by BAL. At the 48 h sampling point, the majority of this reduction was accounted for by the marked inhibition of eosinophil recruitment (Figure 4b).

Systemic Exposure. The PK behavior of a range of compounds was measured in rats to assess the extent of any systemic exposure which might lead to side effects. Systemic exposure following drug delivery by inhalation can arise from the portion of the dose that is inadvertently swallowed, creating the potential for both on- and off-target systemic effects, so part of our design strategy was to minimize oral absorption. Further studies are planned to assess the extent of systemic exposure directly from the lung.

For existing antiasthma medicines, the systemic effects being avoided are primarily on-target actions. One approach to avoid systemic effects is to introduce a metabolic “soft-spot” so that biotransformation occurs predictably to an inactive metabolite. In this program, the therapeutic target is nonhuman and it is found only in the airways after inhalation, so systemic exposure to ADIs is concerned solely with off-target effects. Although off-targets can be predicted by homology with the therapeutic target, unexpected effects of both the parent drug and its metabolites remain a possibility. Our preferred path was therefore more general, relying on the minimization of free drug levels in plasma by varying PPB and oral bioavailability, alone or in combination. Selected compounds were therefore profiled using a 5 mg/kg oral dose in fed rats with the aim of generating compounds with a free concentration $C_{\max} < 100$ nM. The oral dose was chosen primarily to allow accurate quantitative analysis because in practice the actual dose of an inhaled compound would be >300-fold less. In these studies, a number of compounds showed encouragingly low levels of free drug (Table 4). For the smaller lipophilic analogue 35, high oral bioavailability was observed, but for higher MW analogues such as 38 and 28, the combination of reduced oral bioavailability and high PPB resulted in negligible levels of free drug even at doses more than 2 orders of magnitude higher than those that would be given therapeutically. As anticipated, the quaternization of amines, as demonstrated by compounds 32 and 52, was a particularly effective means of reducing oral absorption.

Table 4. Levels of Exposure Following Oral Dosing to Rats

| compd no. | F % ^a | C_{\max} 5 mg/kg po (nM) | PPB (%) | free conc C_{\max} (nM) |
|-----------|------------------|----------------------------|------------|---------------------------|
| 35 | 90 ± 11 | 1136 ± 268 | 84.5 ± 0.6 | 176 ± 0 |
| 38 | 33 ± 8 | 732 ± 3 | 96.0 ± 0.2 | 29 ± 0 |
| 28 | 20 ± 1 | 663 ± 26 | 98.0 ± 0.1 | 11 ± 0 |
| 52 | 0 ± 0 | 7 ± 2 | 63.5 ± 0.7 | 3 ± 0 |
| 32 | 1 ± 0 | 20 ± 13 | 61.2 ± 0.2 | 8 ± 0 |

^aBioavailability based upon comparison with a 1 mg/kg (1–2 μmol/kg according to compound) iv dose. Data are mean ± SE ($n = 3$ animals/compound).

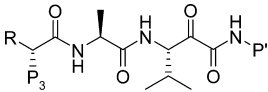
Further Optimization. The P₄ and P' substituents were subsequently varied to generate a wider range of compounds with the desired potency/selectivity profile (Table 5). The most promising compounds from the entire compound set were subsequently evaluated in depth using in vivo models.

With low systemic exposure, excellent Der p 1 potency and selectivity, high aqueous solubility, and efficacy beyond 6 h, compounds 32 and 52 exemplify one approach to the design of Der p 1 inhibitors (Table 6). Furthermore, Table 6 demonstrates that by using Der p 1 as the chemical design template and the screening target, it was possible to obtain potent inhibitors of an orthologous group 1 HDM allergen from another HDM species, *D. farinae*, confirming their behavior as a single drug target.

CONCLUSION

This program has identified compounds which create an innovative approach to the treatment of allergic asthma. Unusually, the therapeutic target is nonhuman, it is contacted by inhalation, and its engagement with an inhibitor is extracellular, features which are attractive for chemical design. Starting from leads which act irreversibly, we designed potent, reversible inhibitors of Der p 1. Clinically, Der p 1 is widely considered to be archetypal of group 1 allergens from all HDM species and is used as a surrogate measure of environmental exposure to HDM generally. Immunological reactivity to group 1 HDM allergens is globally prevalent and is found in >90% of patients who are allergic to HDM.^{9,15,42} Collectively, these strategic factors make HDM the major domestic trigger of asthma attacks. Our work validates Der p 1 as an archetype for drug design because we were able to demonstrate the principle that leading compounds were equally effective as inhibitors of the orthologous group 1 allergen from another clinically significant HDM species. The cysteine peptidase activity of Der p 1 and, by inference, other group 1 HDM allergens, is of functional significance to the development of allergic disease through general mechanisms which are targeted by these new inhibitors.²² We call the new compounds “allergen delivery inhibitors” to reflect the sentinel events which are blocked by this intervention.

Candidate ADIs were generated by optimizing interactions with the binding pockets and improvements in selectivity were largely achieved by modifying the substituents interacting at P₃ and P'. In the case of the P₃ position, this can be rationalized by the presence of a more open substrate-binding pocket in Der p 1. Additionally, by modifying their terminal groups, we have demonstrated that Der p 1 inhibitors with a wide range of physicochemical properties can be identified, allowing the impact of such properties on in vivo efficacy to be explored. These studies show that lipophilic compounds, and those

Table 5. Further Optimization of the P₄ and P' Groups with Respect to Der p 1 Potency and Selectivity over Cat B


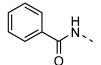
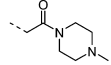
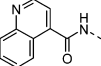
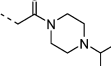
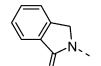
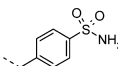
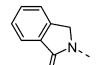
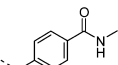
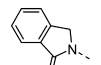
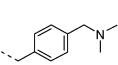
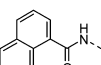
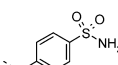
| Cpd # | R | P ₃ | P' | Der p 1 IC ₅₀ (nM) | Cat B IC ₅₀ (nM) |
|-------|--|----------------|---|-------------------------------|-----------------------------|
| 54 |  | Adamantan-1-yl |  | 5 ± 0 | >2500 |
| 55 |  | <i>t</i> -Bu |  | 3 ± 0 | >2500 |
| 56 |  | <i>t</i> -Bu |  | 3 ± 0 | 3049 ± 1129 |
| 57 |  | <i>t</i> -Bu |  | 4 ± 0 | 3571 ± 850 |
| 58 |  | <i>t</i> -Bu |  | 4 ± 0 | 2847 ± 662 |
| 59 |  | <i>t</i> -Bu |  | 4 ± 0 | 532 ± 136 |

Table 6. Overall Profile of Compounds 32 and 52

| compd no. | Der p 1 ^a (nM) | Der f 1 ^a (nM) | Cat B ^a (nM) | Cat S ^a (nM) | F (%) ^b | reduction in innate BAL eosinophil response (%) ^c |
|-----------|---------------------------|---------------------------|-------------------------|-------------------------|--------------------|--|
| 32 | 6 ± 1 | 4 ± 0 | 274 ± 44 | 108 ± 17 | 0.6 ± 0.2 | 58.7 ± 11.3 ^d |
| 52 | 17 ± 2 | 11 ± 1 | >2500 | 114 ± 7 | 0 ± 0 | 65.6 ± 7.1 ^d |

^aIn vitro enzymatic assays were performed as described in Experimental Section. ^bBioavailability was determined in groups of 3 nonfasted Sprague–Dawley rats as described in Supporting Information. Data are mean ± SE (*n* = 3). ^cCompounds were dosed as intratracheal aerosols 6 h prior to challenge of groups of 10 animals with the HDM allergen mixture. Dose delivered to each animal was ~40 μg/kg at a drug:target molar ratio of 50:1. ^dMean ± SE, in groups of 10 animals. *P* < 0.001 compared to control (one-way ANOVA).

incorporating a quaternary ammonium group, display superior in vivo efficacy in a rodent HDM allergen challenge model. A number of the compounds have a high-melting crystalline form and show low levels of exposure when dosed orally, two further properties which are desirable in an inhaled drug.

Epidemiological studies highlight the importance of HDM allergy as both an asthma trigger and a facilitator of allergic sensitization generally.⁴² This central importance of HDM and their allergens accords with the sentinel roles that innate mechanisms play in the development of allergic asthma through the activation of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) which are linked to the expression of TH₂-polarized adaptive immunity.¹⁵ HDM, with their environmental pervasiveness and extensive repertoire of allergens of diverse biological activity, are understandably decisive regulators of innate responses operating through such mechanisms. While the exact nature of the collateral priming mechanisms activated by HDM exposure is incompletely understood, compelling evidence implicates cysteine peptidase group 1 allergens as key components.^{6,16,18,22–24,37} This is supported by our in vivo experi-

ments where we show, for the first time, that the targeted inhibition of a group 1 allergen by suitably optimized compounds substantially reduces cellular inflammation following challenge with a mixture comprising more than 20 different allergens. This suggests that ADIs have the potential to influence inter alia a broad spectrum of innate pathways which form the general mechanism which underpins the development, maintenance, and, ultimately, escalation of allergic asthma. The emergence of promising candidates from this program, including one nominated for development, will enable evaluation of a new therapeutic approach to allergic asthma based upon treating its root cause rather than by amelioration of its symptoms.

EXPERIMENTAL SECTION

The syntheses of key compounds are described below. All commercially available solvents and reagents were used without further purification unless otherwise noted. NMR spectra were measured with a Bruker DRZ 400 MHz spectrometer; chemical shifts are expressed in ppm and are aligned relative to the residual solvent peak, e.g., 2.5 ppm for DMSO. Coupling constants (*J*) are recorded in Hz. The purity of test compounds was determined by reverse-phase

LC-MS using an analytical C18 column (Phenomenex Luna C₁₈ (2) 150 mm × 4.6 mm, 5 μm), using a diode array detector and an A:B gradient starting from 95% A:5%B at a flow rate of 1.5 mL/min, where eluent A was 0.1% formic acid/H₂O and eluent B was 0.1% formic acid/MeCN or 0.1% formic acid/MeOH. Mass spectra were obtained using a Waters ZQ4000 single quadrupole or a Micromass Ultima triple quadrupole mass spectrometer. Silica gel (60 Å, 40–63 μm, Fisher) or cartridges (Biotage snap cartridges KP-SIL or Isolute Si-II cartridges) were used for flash chromatography. Routine analytical thin layer chromatography was performed on precoated plates (Alugram, SILG/UV254). Reverse-phase preparative HPLC was carried out on a Waters ZQ instrument using mass-directed purification on a preparative C₁₈ column (Phenomenex Luna C₁₈ (2), 100 mm × 21.2 mm, 5 μm). Depending upon the retention time and the degree of separation of the desired product from any impurities an A:B gradient was employed starting from high %A low %B at a flow rate of 20 mL/min. The following combinations of A and B were typically used: A = H₂O + 0.1% formic acid, B = MeOH + 0.1% formic acid, or A = H₂O + 0.1% TFA, B = MeCN + 0.1% TFA, or A = 10 mM NH₄HCO₃ (aq), B = MeOH. All compounds tested were at least 95% pure by LC-MS unless otherwise stated.

In all cases pyruvamide inhibitors of Der p 1 were synthesized by oxidation of the corresponding α-hydroxyamide (7) using the same general procedure.

General Procedure I: Oxidation of α-Hydroxyamides (7) to Pyruvamides. To a stirred solution of 7 (1 equiv) in anhydrous DCM (1 mL/25–250 mg of alcohol) and anhydrous DMF (10–35% v/v depending upon solubility) at ambient temperature was added Dess–Martin periodinane (1.6 equiv) in portions. The reaction mixture was stirred at ambient temperature and monitored by LC-MS until full conversion to product pyruvamide had occurred (typically 1 h to 1 day). Where necessary, additional Dess–Martin periodinane was added to complete the oxidation. The reaction mixture was quenched by addition of saturated NaHCO₃ (aq) (1 volume) and Na₂S₂O₃ (aq, 10% w/v). The mixture was stirred for ~30 min, diluted with ethyl acetate (10 volumes), and washed with saturated NaHCO₃ (aq) (2 × 5 volumes), deionized water (5 volumes), and brine (5 volumes). The organic layer was subsequently dried over MgSO₄, filtered, and evaporated. Purification by reverse-phase preparative HPLC was generally followed by lyophilization to give the desired pyruvamide.

The synthesis of pyruvamide compounds via routes A, B, C, or D required the synthesis of intermediates 6a–l, 8a–c, 9a, and 15a–i. The syntheses of these compounds are described in the Supporting Information.

N-[(S)-1-[(S)-1-((S)-1-Cyclohexylaminooxalyl-pentylcarbamoyl)-ethylcarbamoyl]-2-phenyl-ethyl]-benzamide (5). Compound 5 was made using Route A:

N-[(S)-1-[(S)-1-((S)-1-Formyl-pentylcarbamoyl)-ethylcarbamoyl]-2-phenyl-ethyl]-benzamide (14a). To a solution of acid 6a (2.0 g, 5.9 mmol) in anhydrous THF (50 mL) at –40 °C was added *N*-methylmorpholine (2.0 mL, 18.2 mmol) and *i*-BuOCOCi (0.8 mL, 6.4 mmol), and the reaction mixture was stirred for 1 h at –40 °C. A solution of (S)-2-amino-1-hexanol (757 mg, 6.4 mmol) in dry THF (5 mL) was then added dropwise and the reaction mixture stirred at –40 °C for 6 h before warming to room temperature slowly over 1–2 h. The resulting mixture was diluted with EtOAc (150 mL) and washed with saturated NaHCO₃ (aq) (2 × 100 mL) and brine (1 × 100 mL). The organic layer was dried over MgSO₄, filtered, and evaporated to give a crude oil. The oil was purified by flash chromatography on silica, eluting with EtOAc to 30% MeOH/EtOAc to give a white solid (1.6 g; MS [M + H]⁺ 440). The solid was dissolved in anhydrous DCM (40 mL) and anhydrous DMF (8 mL) and Dess–Martin periodinane (1.5 g, 3.4 mmol) was added. The reaction mixture was stirred at ambient temperature for 4 h and monitored by LC-MS; if the reaction had not gone to completion, a further amount of Dess–Martin periodinane was added and the reaction mixture stirred until completion (in this instance 450 mg, 1.1 mmol was added and the reaction mixture stirred for a further 2 h). The solvent was removed by evaporation and the crude mixture redissolved in EtOAc (50 mL). A solution of sodium

thiosulfate in saturated NaHCO₃ (aq) (3 g in 30 mL) was added and the mixture stirred for ~30 min. The organic layer was separated and washed with saturated NaHCO₃ (aq) (2 × 50 mL) and brine (50 mL) then dried over MgSO₄, filtered, and evaporated to yield a pale-yellow oil. Trituration with Et₂O gave 14a as white solid (683 mg, 26%); [M + H]⁺ 438. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 9.40 (1H, s, C(O)H), 8.58 (1H, d, J = 8.3 Hz, NH), 8.33 (1H, d, J = 7.3 Hz, NH), 8.27 (1H, d, J = 7.1 Hz, NH), 7.80–7.75 (2H, m, ArH), 7.54–7.49 (1H, m, ArH), 7.47–7.41 (2H, m, ArH), 7.40–7.35 (2H, m, ArH), 7.29–7.23 (2H, m, ArH), 7.19–7.13 (1H, m, ArH), 4.75–4.68 (1H, m, CHC(O)), 4.41–4.33 (1H, m, CHC(O)), 4.10–4.03 (1H, m, CHC(O)), 3.13 (1H, dd, J = 13.9, 4.0 Hz, 1 of CH₂Ph), 2.98 (1H, dd, J = 13.9, 11.1 Hz, 1 of CH₂Ph), 1.80–1.61 (1H, m), 1.52–1.44 (1H, m), 1.34–1.22 (7H, m), 0.87–0.82 (3H, m).

Compound 5. To a stirred solution of 14a (218 mg, 0.5 mmol) in anhydrous DCM (2 mL) at 0 °C was added cyclohexylisocyanide (74 μL, 0.6 mmol) and pyridine (161 μL, 2.0 mmol) followed by dropwise addition of TFA (74 μL, 1.0 mmol). The reaction mixture was stirred at 0 °C for 10 min and then allowed to warm to ambient temperature. After 4 h at room temperature, analytical LC-MS suggested that considerable starting material remained. Therefore, further cyclohexylisocyanide (61 μL, 0.5 mmol) and TFA (37 μL, 0.5 mmol) was added and the reaction mixture stirred for 18 h. The mixture was diluted with DCM (10 mL), washed with saturated NaHCO₃ (aq) (2 × 15 mL) and brine (15 mL), and the organic layer then dried over MgSO₄, filtered and evaporated. The crude material was oxidized following general procedure I to give compound 5 (76 mg, 27%); [M + H]⁺ 563. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 8.58 (1H, d, J = 8.6 Hz, NH), 8.55 (1H, d, J = 8.4 Hz, NH), 8.28 (1H, d, J = 7.6 Hz, NH), 8.22 (1H, d, J = 7.1 Hz, NH), 7.80–7.74 (2H, m, ArH), 7.55–7.48 (1H, m, ArH), 7.47–7.41 (2H, m, ArH), 7.40–7.35 (2H, m, ArH), 7.30–7.23 (2H, m, ArH), 7.19–7.14 (1H, m, ArH), 5.00–4.94 (1H, m, CHCO), 4.76–4.66 (1H, m, CHCO), 4.42–4.34 (1H, m, CHCO), 3.60–3.50 (1H, m, NHCH(cyclohexyl)), 3.12 (1H, dd, J = 13.8 and 3.4 Hz, 1 of CH₂Ph), 2.96 (1H, dd, J = 13.8 and 11.2 Hz, 1 of CH₂Ph), 1.80–1.40 (7H, m), 1.39–1.00 (12H, m), 0.82 (3H, t, J = 6.9 Hz, CH₃).

((S)-2-Cyano-2-hydroxy-1-isopropyl-ethyl)-carbamic Acid Benzyl Ester (17). To Cbz-Val-OH (16) (50.0 g, 199 mmol), *N,O*-dimethylhydroxylamine hydrochloride (38.8 g, 398 mmol) and EDC·HCl (47.7 g, 249 mmol) in DCM (500 mL) was added DIPEA (87 mL, 497 mmol), and the reaction mixture stirred at ambient temperature for 20 h, after which time it was diluted with DCM (200 mL), washed with 1 M HCl (aq) (3 × 200 mL), 1 M NaOH (aq) (200 mL), saturated NaHCO₃ (aq) (200 mL), and brine (300 mL). The organic layer was dried over MgSO₄, filtered, and the solvent removed under vacuum to give the desired compound as a colorless oil (51.2 g; MS [M + H]⁺ 295). A portion of this (33 g, ~112 mmol) was dissolved in anhydrous THF (300 mL) at –30 to –40 °C, and LiAlH₄ (4.3 g, 113 mmol) was added portionwise over a period of 45 min. The mixture was warmed to 0 °C and stirred for 2 h. The reaction mixture was quenched at 0 °C with 1 M KHSO₄ (330 mL) then 10% w/v Rochelle's salt (aq) (330 mL) was added and the mixture stirred for 20 min then extracted with EtOAc (2 × 700 mL). The combined organic phases were washed with 10% w/v Rochelle's salt (aq) (330 mL) and brine (450 mL), dried over MgSO₄, filtered, and concentrated under vacuum to obtain a clear oil (26.3 g, major peak; [M + H]⁺ 236). The oil was dissolved in MeOH (150 mL) and cooled to 0 °C. A solution of NaHSO₃ (11.9 g, 114 mmol) in H₂O (230 mL) was added and the mixture stirred at 0 °C for 2.5 h. The resulting mixture was added to a solution of NaCN (8.5 g, 174 mmol) in H₂O (150 mL) and EtOAc (450 mL) also at 0 °C. After 1 h, this was allowed to warm to ambient temperature and was stirred for 20 h. The EtOAc layer was separated and the aqueous layer extracted with EtOAc (2 × 500 mL). The combined organic extracts were washed with brine (400 mL), dried over MgSO₄, filtered, and concentrated to give compound 17 (29.7 g, crude, ~ 1:1 mixture of diastereomers) as a clear gummy liquid; [M + H₂O]⁺ 280. The mixture was used without further purification in the synthesis of 18.

(S)-3-tert-Butoxycarbonylamino-2-hydroxy-4-methyl-pentanoic Acid (18). To a solution of **18** (5.1 g, 19.5 mmol) in 1,4-dioxane (90 mL) was added concentrated HCl (aq) (90 mL) and anisole (1.5 equiv), and the mixture was heated to 110 °C for 18 h. The reaction mixture was cooled to ambient temperature and concentrated under vacuum to remove the dioxane. The mixture was then washed with EtOAc, and the residue was further concentrated under vacuum at 40 °C to remove the HCl (aq). Residual water was removed by azeotroping with toluene. The residue was washed with Et₂O (2 × 50 mL) to give a gummy solid. The crude mixture was dissolved in methanol (100 mL), and Et₃N (9.0 mL, 64 mmol) was added. Di-*tert*-butyl dicarbonate (4.7 g, 22 mmol, ~1.1 equiv based upon crude solid) was added portionwise, and the reaction mixture was stirred at ambient temperature for 20 h. The reaction mixture was concentrated in vacuo, and the residue was dissolved in EtOAc (100 mL) and 1N NaOH (aq) (75 mL). The organic phase was separated, and the aqueous phase was washed further with EtOAc (2 × 100 mL) to remove any nonpolar/nonacidic impurities. The aqueous layer was then acidified (pH ~2) with 2 N HCl and extracted with EtOAc (3 × 100 mL). The combined organic phases were dried over MgSO₄, filtered, and concentrated under vacuum to give a white, waxy solid. This was further purified on a Biotage Isolute (IST)-NH₂ cartridge (25 g/150 mL). The cartridge was first equilibrated with MeOH (75 mL), MeCN (75 mL) and EtOAc (75 mL). The crude mixture was then loaded in 5% MeOH/EtOAc (50 mL) and washed with EtOAc (2 × 75 mL) and MeCN (75 mL). The desired mixture of diastereomeric acids was then eluted by washing with MeCN containing 1% formic acid (350 mL). A 1:1 diastereomeric mixture of the desired compounds was obtained, as a white solid, following evaporation of the solvent under vacuum (1.5 g, 27% from compound **16**). **18A**: [M - H]⁻ 246; ¹H NMR ((CD₃)₂SO, 400 MHz): δ 12.4 (1H, br s), 6.46 (1H, d, J = 10.0 Hz), 5.38 (1H, br s), 3.83 (1H, d, J = 6.8 Hz), 3.65–3.59 (1H, m), 1.99–1.91 (1H, m), 1.36 (9H, s), 0.81–0.76 (6H, m). **18B**: MS [M - H]⁻ 246; ¹H NMR ((CD₃)₂SO, 400 MHz): δ 12.44 (1H, br s), 6.21 (1H, d, J = 10.0 Hz), 4.95 (1H, br s), 4.11 (1H, d, J = 1.6 Hz), 3.53–3.47 (1H, m), 1.74 (1H, m), 1.35 (9H, s), 0.91–0.83 (6H, m). The single diastereomer **18A** could be isolated by dissolving the mixture of diastereomers in CHCl₃ and adding *n*-pentane to afford isomer **18A** as a white precipitate that could be collected by filtration.

N-[(S)-1-[(S)-1-((S)-1-Cyclohexylaminooxalyl-2-methyl-propylcarbamoyl)-ethylcarbamoyl]-2,2-dimethyl-propyl]-benzamide (22). Compound **22** was prepared as a white solid from compound **6b** (17 mg, 10%) using a similar procedure to that of compound **5**, with the exception that the aldehyde intermediate was purified by reverse-phase preparative HPLC using a H₂O + 0.1% TFA/MeCN + 0.1% TFA gradient at 50 °C followed by lyophilization to remove the solvent. [M + H]⁺ 515. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 8.53 (1H, d, J = 8.1 Hz, NH), 8.19 (1H, d, J = 7.1 Hz, NH), 7.98 (1H, d, J = 7.8 Hz, NH), 7.88 (1H, d, J = 9.2 Hz, NH), 7.86–7.82 (2H, m, ArH), 7.58–7.52 (1H, m, ArH), 7.50–7.44 (2H, m, ArH), 5.04 (1H, dd, J = 7.8, 5.3 Hz, CHCO), 4.50 (1H, d, J = 9.2 Hz, CHCO), 4.49–4.41 (1H, m, CHCO), 3.61–3.50 (1H, m, NHCH₂(cyclohexyl)), 2.22–2.12 (1H, m, CHMe₂), 1.73–1.63 (4H, m), 1.61–1.52 (1H, m), 1.34–1.19 (7H, m), 1.14–1.02 (1H, m), 1.00 (9H, s, *t*-Bu) 0.89 (3H, d, J = 6.8 Hz, CH₃), 0.81 (3H, d, J = 6.8 Hz, CH₃).

Quinoline-4-carboxylic Acid [(S)-1-[(S)-1-((S)-1-Benzylaminooxalyl-2-methyl-propylcarbamoyl)-ethylcarbamoyl]-2,2-dimethyl-propyl]-amide (27). Compound **27** was prepared as a white solid from compound **6c** (32 mg, 62%) using a similar procedure to that of **22**; [M + H]⁺ 574. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 9.30 (1H, t, J = 6.3 Hz, NH), 8.96 (1H, d, J = 4.3 Hz, ArH), 8.72 (1H, d, J = 9.0 Hz, NH), 8.21 (1H, d, J = 6.8 Hz, NH), 8.12 (1H, d, J = 7.8 Hz, NH), 8.08 (1H, d, J = 7.8 Hz, ArH), 8.05 (1H, dd, J = 8.3 Hz, 0.8 Hz, ArH), 7.84–7.78 (1H, m, ArH), 7.68–7.62 (1H, m, ArH), 7.48 (1H, d, J = 4.3 Hz, ArH), 7.35–7.29 (2H, m, ArH), 7.28–7.22 (3H, m, ArH), 5.06 (1H, dd, J = 7.8, 5.4 Hz, CHCO), 4.47 (1H, d, J = 9.0 Hz, CHCO), 4.54–4.45 (1H, m, CHCO), 4.36 (1H, dd, J = 14.7, 6.3 Hz, 1 of CH₂Ph), 4.30 (1H, dd, J = 14.7, 6.3 Hz, 1 of CH₂Ph), 2.25–2.16

(1H, m, CHMe₂), 1.24 (3H, d, J = 7.1 Hz, CH₃), 1.03 (9H, s, *t*-Bu), 0.91 (3H, d, J = 6.8 Hz, CH₃), 0.84 (3H, d, J = 6.8 Hz, CH₃).

(S)-3-[(S)-2-[(S)-3,3-Dimethyl-2-(1-oxo-1,3-dihydro-isoindol-2-yl)-butyrylamino]-propionylamino]-4-methyl-2-oxo-pentanoic Acid Benzylamide (28). To a solution of **6d** (350 mg, 1.1 mmol) in anhydrous THF (10 mL) at -40 °C was added *N*-methylmorpholine (240 μL, 2.1 mmol) and *i*-BuOCOCl (180 μL, 1.2 mmol) and the reaction mixture stirred for 1 h. A solution of compound **15a** (420 mg, 1.2 mmol) in dry THF (5 mL) and *N*-methylmorpholine (180 μL, 1.6 mmol) was then added dropwise and the reaction mixture stirred at -40 °C for 6 h before warming to room temperature over 1–2 h. The solvent was evaporated in vacuo and the crude residue dissolved in EtOAc (50 mL) then washed with saturated NaHCO₃ (aq) (2 × 50 mL) and brine (50 mL). The organic layer was dried over MgSO₄, filtered, and evaporated to give a white solid. Oxidation using general procedure I gave compound **28** as a white solid (277 mg, 47%); [M + H]⁺ 535. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 9.29 (1H, t, J = 6.5 Hz, NH), 8.52 (1H, d, J = 6.8 Hz, NH), 7.99 (1H, d, J = 7.7 Hz, NH), 7.71 (1H, d, J = 7.6 Hz, ArH), 7.64–7.56 (2H, m, ArH), 7.52–7.46 (1H, m, ArH), 7.35–7.29 (2H, m, ArH), 7.28–7.22 (3H, m, ArH), 5.05 (1H, dd, J = 7.7, 5.5 Hz, CHCO), 5.00 (1H, d, J = 18.4 Hz, 1 from dihydro-isoindolone ring), 4.84 (1H, s, CHCO), 4.76 (1H, d, J = 18.4 Hz, 1 from dihydro-isoindolone ring), 4.40–4.27 (3H, m, CH₂Ph and CHCO), 2.26–2.14 (1H, m, CHMe₂), 1.16 (3H, d, J = 7.1 Hz, CH₃), 1.05 (9H, s, *t*-Bu), 0.92 (3H, d, J = 6.8 Hz, CH₃), 0.83 (3H, d, J = 6.8 Hz, CH₃).

N-[(S)-1-[(S)-1-((S)-1-Cyclohexylaminooxalyl-2-methyl-propylcarbamoyl)-ethylcarbamoyl]-2,2-dimethyl-propyl]-isonicotinamide (29). Compound **29** was prepared as a white solid from compound **6e** (59 mg, 5%) using a similar procedure to that of **22**; [M + H]⁺ 516. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 8.75–8.72 (2H, m, ArH), 8.57 (1H, d, J = 8.3 Hz, NH), 8.38 (1H, d, J = 9.4 Hz, NH), 8.24 (1H, d, J = 7.1 Hz), 8.02 (1H, d, J = 7.8 Hz, NH), 7.81–7.78 (2H, m, ArH), 5.04 (1H, dd, J = 7.8 and 5.3 Hz, CHCO), 4.52 (1H, d, J = 9.4 Hz, CHCO), 4.47–4.40 (1H, m, CHCO), 3.60–3.45 (1H, m, NHCH₂(cyclohexyl)), 2.22–2.12 (1H, m, CHMe₂), 1.72–1.64 (4H, m), 1.61–1.54 (1H, m), 1.34–1.17 (7H, m), 1.12–1.04 (1H, m), 1.00 (9H, s, *t*-Bu), 0.89 (3H, d, J = 6.8 Hz, CH₃), 0.80 (3H, d, J = 6.8 Hz, CH₃).

4-[(S)-1-[(S)-1-((S)-1-Benzylaminooxalyl-2-methyl-propylcarbamoyl)-ethylcarbamoyl]-2,2-dimethyl-propylcarbamoyl]-1,1-dimethyl-piperidinium Formate (32). Compound **32** was prepared in its formate salt form from compounds **6f** and **15a** (19 mg, 20%) using a similar procedure to that of compound **28**, yielding a white solid; [M]⁺ 558. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 9.31 (1H, t, J = 6.3 Hz, NH), 8.42 (1H, s, HC(O)⁻ from formate), 8.25 (1H, d, J = 6.8 Hz, NH), 8.13 (1H, d, J = 9.8 Hz), 8.09 (1H, J = 7.8 Hz, NH), 7.35–7.28 (2H, m, ArH), 7.28–7.21 (3H, m, ArH), 5.05 (1H, dd, J = 7.8, 5.3 Hz, CHCO), 4.41–4.23 (4H, m, CH₂Ph and 2 × CHCO), 3.40–3.20 (4H, m, Me₂N⁺(CH₂)₂-), 3.12 (3H, s, ⁺NCH₃), 3.02 (3H, s, ⁺NCH₃), 2.60–2.55 (1H, m, piperidine ring adjacent to C(O)NH), 2.24–2.14 (4H, m, piperidine ring), 1.18 (3H, d, J = 7.0 Hz, CH₃), 0.93–0.87 (12H, m, *t*-Bu and CH₃), 0.82 (3H, d, J = 6.8 Hz, CH₃).

N-[(S)-1-[(S)-1-((S)-1-Benzylaminooxalyl-2-methyl-propylcarbamoyl)-ethylcarbamoyl]-2,2-dimethyl-propyl]-benzamide (38). Compound **38** was prepared from compounds **6b** and **15a**, as a white solid (170 mg, 50%) using a similar procedure to that of **28**; [M + H]⁺ 523. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 9.24 (1H, t, J = 6.3 Hz, NH), 8.18 (1H, d, J = 7.1 Hz, NH), 8.13 (1H, d, J = 7.8 Hz, NH), 7.88 (1H, d, J = 9.5 Hz, NH), 7.86–7.82 (2H, m, ArH), 7.57–7.52 (1H, m, ArH), 7.50–7.45 (2H, m, ArH), 7.35–7.29 (2H, m, ArH), 7.27–7.22 (3H, m, ArH), 5.03 (1H, dd, J = 7.8, 5.6 Hz, CHCO), 4.50 (1H, d, J = 9.5 Hz, CHCO), 4.49–4.40 (1H, m, CHCO), 4.38–4.26 (2H, m, CH₂Ph), 2.25–2.15 (1H, m, CHMe₂), 1.20 (3H, d, J = 7.1 Hz, CH₃), 1.01 (9H, s, *t*-Bu), 0.89 (3H, d, J = 6.8 Hz, CH₃), 0.82 (3H, d, J = 6.8 Hz, CH₃).

N-[(S)-1-[(S)-1-((S)-2-Methyl-1-[2-(4-methyl-piperazin-1-yl)-2-oxo-ethylaminooxalyl]-propylcarbamoyl]-ethylcarbamoyl]-2-phenyl-ethyl]-benzamide (45). Compound **45** was prepared as its TFA salt and was isolated as a white solid from compound **6a** (16 mg, 14%) using a similar procedure to that of **22**; [M + H]⁺ 607. ¹H NMR

((CD₃)₂SO, 400 MHz): δ 9.80 (1H, s, MeN⁺H), 8.71 (1H, t, J = 5.7 Hz, NH), 8.58 (1H, d, J = 8.3 Hz, NH), 8.32 (1H, d, J = 7.1 Hz, NH), 8.09 (1H, d, J = 7.6 Hz, NH), 7.79–7.75 (2H, m, ArH), 7.54–7.49 (1H, m, ArH), 7.47–7.41 (2H, m, ArH), 7.40–7.36 (2H, m, ArH), 7.29–7.23 (2H, m, ArH), 7.19–7.13 (1H, m, ArH), 5.08 (1H, dd, J = 7.6, 5.1 Hz, CHCO), 4.75–4.68 (1H, m, CHCO), 4.50–4.30 (2H, m, CHCO + 1 other), 3.15–2.90 (3H, m), 3.50–3.30 (4H, m partially obscured by HOD residual), 3.15–2.90 (4H, m, CH₂Ph + 2 others), 2.82 (3H, s, NCH₃), 2.26–2.16 (1H, m, CHMe₂), 1.26 (3H, d, J = 7.1 Hz, CH₃), 0.91 (3H, d, J = 6.8 Hz, CH₃), 0.83 (3H, d, J = 6.8 Hz, CH₃).

N-((S)-1-((S)-1-((S)-2-Methyl-1-(2-morpholin-4-yl)-2-oxo-ethylaminooxalyl)-propylcarbamoyl)-ethylcarbamoyl)-2-phenylethyl)-benzamide (47). Compound 47 was prepared as a white solid from compound 6a (8 mg, 5%) using a similar procedure to that of 22; [M + H]⁺ 594. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 8.62 (1H, t, J = 5.8 Hz, NH), 8.58 (1H, d, J = 8.3 Hz, NH), 8.32 (1H, d, 7.3 Hz, NH), 8.08 (1H, d, J = 7.8 Hz, NH), 7.79–7.75 (2H, m, ArH), 7.54–7.48 (1H, m, ArH), 7.46–7.41 (2H, m, ArH), 7.40–7.36 (2H, m, ArH), 7.29–7.23 (2H, m, ArH), 7.18–7.13 (1H, m, ArH), 5.06 (1H, dd, J = 7.8, 5.3 Hz, CHCO), 4.75–4.68 (1H, m, CHCO), 4.50–4.40 (1H, m, CHCO), 4.04 (1H, dd, J = 16.7, 5.8, 1 of CH₂CO-morpholine), 3.98 (1H, dd, J = 16.7, 5.8, 1 of CH₂CO-morpholine), 3.60–3.52 (4H, m, morpholine), 3.45–3.39 (4H, m, morpholine), 3.12 (1H, dd, J = 13.6, 3.4 Hz, 1 of CH₂Ph), 2.97 (1H, dd, J = 13.6, 11.3 Hz, 1 of CH₂Ph), 2.27–2.17 (1H, m, CHMe₂), 1.26 (3H, d, J = 7.1 Hz, CH₃), 0.90 (3H, d, J = 6.8 Hz, CH₃), 0.83 (3H, d, J = 7.1 Hz, CH₃).

4-(2-((S)-3-((S)-2-((S)-2-Benzoylamino-3-phenyl-propionylamino)-propionylamino]-4-methyl-2-oxo-pentanoylamino)-acetyl)-1,1-dimethyl-piperazin-1-ium Formate (52). Compound 52 was prepared as its formate salt and isolated as a white solid (40 mg, 37%) from compounds 6a and 15b using a similar procedure to that of 28; [M]⁺ 621. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 8.78 (1H, t, J = 5.7 Hz, NH), 8.69 (1H, d, J = 8.6 Hz, NH), 8.49 (1H, d, J = 7.3 Hz, NH), 8.47 (1H, s, HC(O)O⁻), 8.15 (1H, d, J = 7.6 Hz, NH), 7.82–7.76 (2H, m, ArH), 7.534–7.48 (1H, m, ArH), 7.46–7.41 (2H, m, ArH), 7.40–7.35 (2H, m, ArH), 7.29–7.23 (2H, m, ArH), 7.18–7.13 (1H, m, ArH), 5.07 (1H, dd, J = 7.6, 5.2 Hz, CHCO), 4.76–4.68 (1H, m, CHCO), 4.49–4.39 (1H, m, CHCO), 4.12 (1H, dd, J = 16.9, 5.7 Hz, 1 of CH₂CO), 4.07 (1H, dd, J = 16.9, 5.7 Hz, 1 of CH₂CO), 3.86–3.74 (4H, m, piperazine ring), 3.44–3.34 (2H, m, piperazine ring), 3.20–3.09 (8H, m, including N⁺(CH₃)₂), 3.04–2.92 (2H, m), 2.26–2.16 (1H, m, CHMe₂), 1.27 (3H, d, J = 7.1 Hz, CH₃), 0.91 (3H, d, J = 6.8 Hz, CH₃), 0.83 (3H, d, J = 6.8 Hz, CH₃).

Naphthalene-1-carboxylic Acid [(S)-1-((S)-1-((S)-2-Methyl-1-[2-(4-methyl-piperazin-1-yl)-2-oxo-ethylaminooxalyl]-propylcarbamoyl)-ethylcarbamoyl)-2-phenylethyl]-amide (53). Compound 53 was prepared as its TFA salt and was isolated as a white solid (30 mg, 18%) from compounds 6g and 15c using a similar procedure to that of 28; [M + H]⁺ 657. ¹H NMR ((CD₃)₂SO, 400 MHz): δ 9.80 (1H, br s, MeN⁺H), 8.76–8.69 (2H, m), 8.29 (1H, d, J = 7.1 Hz), 8.17 (1H, d, J = 7.6 Hz), 7.97 (1H, d, J = 8.3 Hz), 7.93 (1H, d, J = 8.1 Hz), 7.74 (1H, d, J = 8.1 Hz), 7.54–7.47 (2H, m, ArH), 7.44–7.36 (4H, m, ArH), 7.35–7.29 (2H, m, ArH), 7.29–7.22 (1H, m, ArH), 5.09 (1H, dd, J = 7.6, 5.0 Hz, CHCO), 4.90–4.82 (1H, m, CHCO), 4.57–4.47 (1H, m, CHCO), 4.46–4.30 (1H, m), 4.22–3.92 (3H, m), 3.30–2.78 (7H, m), 2.79 (3H, s, NCH₃), 2.27–2.17 (1H, m, CHMe₂), 1.30 (3H, d, J = 7.1 Hz, CH₃), 0.91 (3H, d, J = 6.8 Hz, CH₃), 0.84 (3H, d, J = 6.8 Hz, CH₃) also 1 × H under HOD 3.40–3.20 ppm.

Group 1 HDM Peptidase Allergen Inhibition Assays. Automated screening of inhibitory activity toward group 1 HDM peptidase allergens was performed as described elsewhere^{25,43} with the minor modification that Der p 1 or Der f 1 were incubated with inhibitors for 20 min prior to initiation of reactions by addition of substrate. In initial screening, the activity of each compound was measured using a series of 10 doubling dilutions from a starting concentration of 50 μ M. Compounds showing optimizable activity were cherry-picked for multiple (up to six) rounds of rescreening to confirm activity and stability and to determine IC₅₀ values over a range

of concentrations that were compound-directed by initial screening. Assays were performed in triplicate for calculation of mean \pm SE IC₅₀ values.

Cathepsin Inhibition Assays. Off-target screening adopted the same principles as target potency determination. Inhibition of Cat B was measured as described with modifications.²⁵ Reaction mixtures comprised 10 μ L of human liver Cat B (0.5 nM final concentration, Merck Chemicals Ltd. UK) preactivated by 2.5 mM 1,4-dithioerythritol (DTE, Sigma-Aldrich) at 37 °C for 10 min, 70 μ L of reaction buffer (0.1 M NaAc-HAc, pH 4.5, 0.2 M NaCl), and 10 μ L of 22.5 mM DTE (2.5 mM final concentrations). Reactions were initiated by adding 10 μ L of 59 μ M substrate ABZ-Gly-Ile-Val-Arg-Ala-Lys-DNP-OH (Merck Chemicals Ltd., UK) dissolved in reaction buffer. Progress curves were followed at 30 °C by detection of fluorescence at 320/420 nm (excitation/emission).

To test inhibitory potential against Cat S, reaction mixtures comprised 70 μ L of reaction buffer (0.1 M sodium phosphate/2 mM EDTA, pH 7.4), 10 μ L of recombinant human Cat S (25 nM in reaction buffer and 10 μ L DTT (2 mM final concentration). Reactions were initiated by addition of 10 μ L of substrate (Z-Phe-Arg-AMC, 20 μ M final concentration in assay). Reactions were performed at 30 °C and progress followed by excitation/emission at 320/420 nm.

In all cases, for reactions involving inhibitors, the amount of reaction buffer was adjusted to 60 μ L, the inhibitor added as a 10 μ L aliquot, and incubated for 20 min with the enzyme prior to reaction start.

Counter-screening against a broader range of proteases, with exemplification for compound 38, is described in Appendix 7 of the Supporting Information.

Allergen Challenge. Brown Norway strain rats (male, 300–350 g at time of allergen challenge, 12–16 weeks old, Harlan UK Ltd.) were used to explore the pharmacological properties of ADIs. To examine the relationship between the physicochemical properties of compounds and their duration of protection through the intended mechanism of action, animals were used without prior sensitization to HDM so that allergen challenge activated only IgE-independent innate mechanisms.

For other studies, rats were actively sensitized to a natural mixture of HDM allergens prepared from laboratory cultures of *Dermatophagoides pteronyssinus*. Animals were sensitized by the ip route in the absence of additional adjuvant on day 0, 7, and 14. This protocol is known to result in the development of allergic sensitization as judged by the appearance of allergen-specific IgE. For standardization purposes, sensitization mixtures were normalized to contain 10 μ g Der p 1 of known catalytic activity, as previous studies indicated that this yielded satisfactory adjuvantless responses. Negative control groups comprised naïve, unsensitized animals or those sham-sensitized with vehicle solution.

Test ADIs were delivered as intratracheal aerosols of 25–30 μ m mass median diameter from a Penn-Century IA-1B microsprayer at various times prior to allergen challenge. Allergen or vehicle challenge was similarly delivered by Penn-Century microsprayer on day 21 of the protocol. These procedures were conducted under anesthesia with isoflurane in oxygen. Test ADIs were generally dosed in a constant 50:1 molar ratio to the Der p 1 content of the allergen challenge mixture. In practice, this resulted in doses of 35–45 μ g/kg being delivered to animals, except in the case of compound 38, where the maximum dose used was 12 μ g/kg (20 nmol/kg).

All in vivo studies were conducted within the jurisdiction of, and in accordance with, the UK Animals (Scientific Procedures) Act, 1986, in an AAALAC-accredited facility. Groups typically comprised 10 animals randomly assigned to treatment. Treatments were coded prior to formulation so that staff responsible for dosing were unaware of test substance identity.

Pulmonary Leukocyte Accumulation. Animals were killed by sodium pentobarbital overdose 48 h after allergen challenge because this is the optimal sampling point to assess the recruitment of eosinophils (our unpublished observations). The airways were lavaged with 3 × 4 mL aliquots of Hanks' Balanced Salt Solution and the recovered cells pooled and counted automatically (ADVIA; Bayer

Healthcare, Diagnostics Division, UK). Differential counts were obtained after the preparation of smears by cyto centrifugation and the staining of methanol-fixed cells with buffered eosin and methylene blue/azur 2 (Speedy-Diff; ClinTech Ltd., Guildford, Surrey UK). Cells were counted by an independent observer using light microscopy under oil immersion ($\times 1000$). Statistical analyses of cell data were made by one-way ANOVA and Holm–Sidak test.

■ ASSOCIATED CONTENT

■ Supporting Information

Synthesis details for intermediates **6a–l**, **8a–c**, **9a**, and **15a–i**; synthesis details for leading subsidiary compounds; sequence overlays of Der p1, Cat B, Cat S, and Cat K; methods for PK studies, PPB measurement, stability assays with rat lung cells, duration of protection profiles for 12 selected compounds in rats, protease-counter screening, XRPD, DSC, and log $D_{7.4}$ determination. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

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■ ABBREVIATIONS USED

\pm SE, plus and minus the standard error on the mean; AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care International; ABz, 2-aminobenzoyl; ADI, allergen delivery inhibitor; AMC, 7-amino-4-methylcoumarin; BAL, bronchoalveolar lavage; BN, Brown Norway; Cat B, cathepsin B; Cat K, cathepsin K; Cat S, cathepsin S; CCL-20, chemokine (C–C motif) ligand 20; Cpd #, compound number; DIPEA, *N,N*-diisopropylethylamine; DNP, 2,4-dinitrophenol; DPI, dry powder inhaler; DSC, differential scanning calorimetry; DTE, 1,4-dithioerythritol; EDC, *N*-ethyl-*N'*-(3-(dimethylamino)propyl)carbodiimide; F12K, Kaighn's modification of Ham's F12 medium; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; HDM, house dust mite; HOBt, 1-hydroxybenzotriazole; IgE, immunoglobulin E; IL, interleukin; MES, 2-(*N*-morpholino)ethanesulfonic acid hydrate; ND, not determined; P₁, P₂, P₃, P₄, and P', refers to the standard Schechter–Berger nomenclature for residues that bind into the S₁, S₂, S₃, S₄, and S' sites on a protease, respectively; t(Bu)Gly, *t*-leucine also known as *t*-butylglycine; TBTU, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; TBDPS, *tert*-butyldiphenylsilyl; TSLP, thymic stromal lymphopoietin; XRPD, X-ray powder diffraction

■ REFERENCES

- (1) Anandan, C.; Nurmatov, U.; van Schayck, O. C.; Sheikh, A. Is the prevalence of asthma declining? Systematic review of epidemiological studies. *Allergy* **2010**, *65*, 152–167.
- (2) Arbes, S. J., Jr.; Cohn, R. D.; Yin, M.; Muilenberg, M. L.; Burge, H. A.; Friedman, W.; Zeldin, D. C. House dust mite allergen in US beds: results from the First National Survey of Lead and Allergens in Housing. *J. Allergy Clin. Immunol.* **2003**, *111*, 408–414.
- (3) Gelber, L. E.; Seltzer, L. H.; Bouzoukis, J. K.; Pollart, S. M.; Chapman, M. D.; Platts-Mills, T. A. Sensitization and exposure to indoor allergens as risk factors for asthma among patients presenting to hospital. *Am. Rev. Respir. Dis.* **1993**, *147*, 573–578.
- (4) Peat, J. K.; Tovey, E.; Toelle, B. G.; Haby, M. M.; Gray, E. J.; Mahmic, A.; Woolcock, A. J. House dust mite allergens. A major risk factor for childhood asthma in Australia. *Am. J. Respir. Crit. Care Med.* **1996**, *153*, 141–146.
- (5) Peat, J. K.; Woolcock, A. J. Sensitivity to common allergens: relation to respiratory symptoms and bronchial hyper-responsiveness in children from three different climatic areas of Australia. *Clin. Exp. Allergy* **1991**, *21*, 573–581.
- (6) Pichavant, M.; Charbonnier, A. S.; Taront, S.; Bricchet, A.; Wallaert, B.; Pestel, J.; Tonnel, A. B.; Gosset, P. Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. *J. Allergy Clin. Immunol.* **2005**, *115*, 771–778.
- (7) Platts-Mills, T. A.; Hayden, M. L.; Chapman, M. D.; Wilkins, S. R. Seasonal variation in dust mite and grass-pollen allergens in dust from the houses of patients with asthma. *J. Allergy Clin. Immunol.* **1987**, *79*, 781–791.
- (8) Platts-Mills, T. A.; Vervloet, D.; Thomas, W. R.; Aalberse, R. C.; Chapman, M. D. Indoor allergens and asthma: report of the Third International Workshop. *J. Allergy Clin. Immunol.* **1997**, *100*, S2–24.
- (9) Platts-Mills, T. A. E. Indoor Allergens. In *Middleton's Allergy: Principles and Practice*, 7th ed.; Mosby Elsevier: Philadelphia, 2009; pp 539–555.
- (10) Smith, J. M.; Disney, M. E.; Williams, J. D.; Goels, Z. A. Clinical significance of skin reactions to mite extracts in children with asthma. *Br. Med. J.* **1969**, *1*, 723–726.
- (11) Sporik, R.; Holgate, S. T.; Platts-Mills, T. A.; Cogswell, J. J. Exposure to house-dust mite allergen (Der p I) and the development of asthma in childhood. A prospective study. *N. Engl. J. Med.* **1990**, *323*, 502–507.
- (12) Squillace, S. P.; Sporik, R. B.; Rakes, G.; Couture, N.; Lawrence, A.; Merriam, S.; Zhang, J.; Platts-Mills, T. A. E. Sensitization to dust mites as a dominant risk factor for asthma among adolescents living in central Virginia. Multiple regression analysis of a population-based study. *Am. J. Respir. Crit. Care Med.* **1997**, *156*, 1760–1764.
- (13) Purello-D'Ambrosia, F.; Gangemi, S.; Merendino, R. A.; Isola, S.; Puccinelli, P.; Parmiani, S.; Ricciardi, L. Prevention of new sensitizations in monosensitized subjects submitted to specific immunotherapy or not. A retrospective study. *Clin. Exp. Allergy* **2001**, *31*, 1295–1302.
- (14) Silvestri, M.; Rossi, G. A.; Cozzani, S.; Pulvirenti, G.; Fasce, L. Age-dependent tendency to become sensitized to other classes of aeroallergens in atopic asthmatic children. *Ann. Allergy Asthma Immunol.* **1999**, *83*, 333–340.
- (15) Stewart, G. A.; Richardson, J. P.; Zhang, J.; Robinson, C. The structure and function of allergens. In *Middleton's Allergy: Principles and Practice*, 8th ed.; Adkinson, N. F., Bochner, B. S., Burks, A. W., Busse, W. W., Holgate, S. T., Lemanske, R. F., O'Hehir, R. E., Eds.; Elsevier Saunders: Philadelphia, 2014; pp 398–429.
- (16) Asokanathan, N.; Graham, P. T.; Stewart, D. J.; Bakker, A. J.; Eidne, K. A.; Thompson, P. J.; Stewart, G. A. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J. Immunol.* **2002**, *169*, 4572–4578.
- (17) Brown, A.; Farmer, K.; MacDonald, L.; Kalsheker, N.; Pritchard, D.; Haslett, C.; Lamb, J.; Sallenave, J. M. House dust mite Der p 1

downregulates defenses of the lung by inactivating elastase inhibitors. *Am. J. Respir. Cell Mol. Biol.* **2003**, *29*, 381–389.

(18) Comoy, E. E.; Pestel, J.; Duez, C.; Stewart, G. A.; Vendeville, C.; Fournier, C.; Finkelman, F.; Capron, A.; Thyphronitis, G. The house dust mite allergen, *Dermatophagoides pteronyssinus*, promotes type 2 responses by modulating the balance between IL-4 and IFN- γ . *J. Immunol.* **1998**, *160*, 2456–2462.

(19) Fattouh, R.; Pouladi, M. A.; Alvarez, D.; Johnson, J. R.; Walker, T. D.; Goncharova, S.; Inman, M. D.; Jordana, M. House dust mite facilitates ovalbumin-specific allergic sensitization and airway inflammation. *Am. J. Respir. Crit. Care Med.* **2005**, *172*, 314–321.

(20) Gough, L.; Sewell, H. F.; Shakib, F. The proteolytic activity of the major dust mite allergen Der p 1 enhances the IgE antibody response to a bystander antigen. *Clin. Exp. Allergy* **2001**, *31*, 1594–1598.

(21) King, C.; Brennan, S.; Thompson, P. J.; Stewart, G. A. Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium. *J. Immunol.* **1998**, *161*, 3645–3651.

(22) Robinson, C.; Zhang, J.; Newton, G. K.; Perrior, T. R. Nonhuman targets in allergic lung conditions. *Future Med. Chem.* **2013**, *5*, 147–161.

(23) Takai, T.; Kato, T.; Ota, M.; Yasueda, H.; Kuhara, T.; Okumura, K.; Ogawa, H. Recombinant Der p 1 and Der f 1 with in vitro enzymatic activity to cleave human CD23, CD25 and α_1 -antitrypsin, and in vivo IgE-eliciting activity in mice. *Int. Arch. Allergy Immunol.* **2005**, *137*, 194–200.

(24) Wan, H.; Winton, H. L.; Soeller, C.; Tovey, E. R.; Gruenert, D. C.; Thompson, P. J.; Stewart, G. A.; Taylor, G. W.; Garrod, D. R.; Cannell, M. B.; Robinson, C. Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions. *J. Clin. Invest.* **1999**, *104*, 123–133.

(25) Zhang, J.; Hamilton, J. M.; Garrod, D. R.; Robinson, C. Interactions between mature Der p 1 and its free prodomain indicate membership of a new family of C1 peptidases. *Allergy* **2007**, *62*, 1302–1309.

(26) Zhang, J.; Zuo, J.; Yin, Y.; Garrod, D. R.; Robinson, C. Identification of Der p 1 cleavage sites in the first adhesion domain of claudin-1. *Am. J. Respir. Crit. Care Med.* **2009**, *179*, A5173.

(27) Jahnsen, F. L.; Moloney, E. D.; Hogan, T.; Upham, J. W.; Burke, C. M.; Holt, P. G. Rapid dendritic cell recruitment to the bronchial mucosa of patients with atopic asthma in response to local allergen challenge. *Thorax* **2001**, *56*, 823–826.

(28) Möller, G. M.; Overbeek, S. E.; Helden-Meeuwssen, C. G.; Van Haarst, J. M.; Prens, E. P.; Mulder, P. G.; Postma, D. S.; Hoogsteden, H. C. Increased numbers of dendritic cells in the bronchial mucosa of atopic asthmatic patients: downregulation by inhaled corticosteroids. *Clin. Exp. Allergy* **1996**, *26*, 517–524.

(29) Zhang, J.; Richardson, J.; Chen, J.; Francis, N.; Garrod, D.; Perrior, T.; Newton, G.; Jenkins, K.; Beevers, R.; Major, M.; Stewart, M.; Robinson, C. A novel Der p 1 inhibitor ADZ 51,457 inhibits eosinophil recruitment and chemokine release following house dust mite allergen challenge in brown Norway rats. *Am. J. Respir. Crit. Care Med.* **2012**, *185*, A2792.

(30) Prefontaine, D.; Nadigel, J.; Chouiali, F.; Audusseau, S.; Semlali, A.; Chakir, J.; Martin, J. G.; Hamid, Q. Increased IL-33 expression by epithelial cells in bronchial asthma. *J. Allergy Clin. Immunol.* **2010**, *125*, 752–754.

(31) Ying, S.; O'Connor, B.; Ratoff, J.; Meng, Q.; Mallett, K.; Cousins, D.; Robinson, D.; Zhang, G.; Zhao, J.; Lee, T. H.; Corrigan, C. Thymic stromal lymphopoietin expression is increased in asthmatic airways and correlates with expression of Th2-attracting chemokines and disease severity. *J. Immunol.* **2005**, *174*, 8183–8190.

(32) Moffatt, M. F.; Gut, I. G.; Demenais, F.; Strachan, D. P.; Bouzigon, E.; Heath, S.; von Mutius, E.; Farrall, M.; Lathrop, M.; Cookson, W. O. A large-scale, consortium-based genomewide association study of asthma. *N. Engl. J. Med.* **2010**, *363*, 1211–1221.

(33) Robinson, C.; Zhang, J.; Garrod, D. R.; Perrior, T. R.; Newton, G. K.; Jenkins, K.; Beevers, R. E.; Kimberley, M. R.; Stewart, M. R.

Pyruvamide compounds as inhibitors of dust mite group 1 peptidase allergen and their use. PCT Int. Appl. WO 2011/089396 A2, 2011.

(34) Billson, J.; Clark, J.; Conway, S. P.; Hart, T.; Johnson, T.; Langston, S. P.; Ramjee, M.; Quibell, M.; Scott, R. K. The design and synthesis of inhibitors of the cysteinyl protease, Der p 1. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 993–998.

(35) Marcaccini, S. Post condensation modifications of the Passerini and Ugi reaction. In *Multicomponent Reactions*; Zhu, J., Bienayme, H., Marcaccini, S., Torroba, T., Eds.; Wiley-VCH: New York, 2005; Vol. 1, pp 41–47.

(36) Chan, W. C.; White, P. Basic Procedures. In *Fmoc Solid Phase Peptide Synthesis: A Practical Approach*; Chan, W. C., White, P., Eds.; Oxford University Press: New York, **2000**; Vol. 1, pp 41–76.

(37) Boyd, M. J.; Crane, S. N.; Robichaud, J.; Scheigetz, J.; Black, C.; Chauret, N.; Wang, C.; Masse, F.; Oballa, R. Investigation of ketone warheads as alternatives to the nitrile for the preparation of potent and selective cathepsin K inhibitors. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 675–679.

(38) Chen, J. C.; Uang, B. J.; Lyu, P. C.; Chang, J. Y.; Liu, K. J.; Kuo, C. C.; Hsieh, H. P.; Wang, H. C.; Cheng, C. S.; Chang, Y. H.; Chang, M. D. T.; Chang, W. C. W.; Lin, C. C. Design and synthesis of α -ketoamides as cathepsin S inhibitors with potential applications against tumour invasion and angiogenesis. *J. Med. Chem.* **2010**, *53*, 4545–4549.

(39) Liu, H.; Tully, D. C.; Epple, R.; Bursulaya, B.; Li, J.; Harris, J. C.; William, J. A.; Russo, R.; Tumanut, C.; Roberts, M. J.; Alper, P. B.; He, Y.; Karanewsky, D. S. Design and synthesis of arylaminoethyl amides as noncovalent inhibitors of cathepsin S. Part 1. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4979–4984.

(40) Brown, A. D.; Bunnage, M. E.; Glossop, P. A.; James, K.; Jones, R.; Lane, C. A. L.; Lewthwaite, R. A.; Mantell, S. A.; Perros-Huguet, C. P.; Price, D. A.; Trevethick, M.; Webster, R. The discovery of long acting β_2 -adrenoreceptor agonists. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 4012–4015.

(41) Milan, D. S.; Bunnage, M. E.; Burrows, J. L.; Butcher, K. J.; Dodd, P. G.; Evans, T. J.; Fairman, D. A.; Hughes, S. J.; Kilty, I. C.; Lemaitre, A.; Mahnke, A.; Mathias, J. P.; Philip, J.; Smith, R. T.; Stefaniak, M. H.; Yeadon, M.; Philips, C. Design and synthesis of inhaled p38 inhibitors for the treatment of chronic obstructive pulmonary disease. *J. Med. Chem.* **2011**, *54*, 7797–7814.

(42) Platts-Mills, T. A. E. Indoor Allergens. In *Middleton's Allergy: Principles and Practice*, 8th ed.; Adkinson, N. F., Bochner, B. S., Burks, A. W., Busse, W. W., Holgate, S. T., Lemanske, R. F., O'Hehir, R. E., Eds.; Elsevier Saunders: Philadelphia, 2014; pp 453–469.

(43) Zhang, J.; Saint-Remy, J. M.; Garrod, D. R.; Robinson, C. Comparative enzymology of native and recombinant house dust mite allergen Der p 1. *Allergy* **2009**, *64*, 469–477.