Available online at www.sciencedirect.com
SCIENCE DIRECT
European Journal of Medicinal Chemistry 41 (2006) 950–969
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
http://france.elsevier.com/direct/ejmech

Original article

Substituted 2-phenyl-benzimidazole derivatives: novel compounds that suppress key markers of allergy

Mark L. Richards [a], Shirley Cruz Lio [a], Anjana Sinha [a], Homayon Banie [a], Richard J. Thomas [a], Michael Major [b], Mark Tanji [a], Jagadish C. Sircar [a]

[a] *Avanir Pharmaceuticals, 11388 Sorrento Valley Road, San Diego, CA 92121, USA*
[b] *Chembridge-Major Inc., W130 N10497 Washington Avenue, Germantown, WI 53022, USA*

Received in revised form 9 March 2006; accepted 23 March 2006
Available online 02 May 2006

Abstract

The pharmacotherapy of allergy and asthma has traditionally focused on the effecter molecules of the allergic cascade, while neglecting targets that play an early role in their development. Reasoning that IgE is central to the expansion of atopic diseases, we identified and extended a novel family of 2-(substituted phenyl)-benzimidazole inhibitors of IgE response. Pharmacological activity depends on an intact phenylbenzimidazole-bis-amide backbone, and is optimized by the presence of lipophilic terminal groups composed of either bis cycloalkyl or combinations of aliphatic and halogen-substituted aromatic groups. These compounds also inhibit IL-4 and IL-5 responses in T cells and CD23 expression on B cells, with potencies that parallel their inhibition of IgE. The broad profile of these compounds thus underscores their potential for treating the multifarious pathology of asthma.

Keywords: 2-(Substituted phenyl)-benzimidazoles; CD23; IL-4; IL-5; IgE

1. Introduction

Allergic disorders such as rhinitis, sinusitis, dermatitis, asthma, and food allergy are becoming one the most common and costly of human diseases [1,2]. One popular theory for their rapid expansion in industrialized countries implicates the diminished microbial exposure resulting in the loss of Th1 cell polarization and, ultimately, Th2 cell expansion [3–6]. Despite the near epidemic growth of asthma, however, there has been a paucity of novel pharmacotherapeutic approaches for the treatment of allergy and asthma introduced in the last 30 years. Indeed, the favored strategy has been to develop improved versions of existing drugs such as anti-histamines.

The complexity of allergic disease, particularly asthma, presents a daunting problem for the development of effective medicines. Hypersensitivity of the immune system to a specific antigen (allergen) plays a central role in the initiation of asthma and allergic rhinitis. Key components of this process include Th2 lymphocytes, which are a major cellular infiltrate in asthmatic lung [7,8], and the antibody immunoglobulin E (IgE), which is over produced in the majority of people who suffer from allergies [9–14]. However, numerous other components have also been implicated as indeed control of the IgE response does not always translate to control of asthma [15–17]. For example, the low affinity receptor for IgE (CD23) has been reported to have direct effects on IgE regulation, antigen presentation, and airway hyper-responsiveness [18–26]. Interleukin-4 (IL-4) and IL-13 also are required for IgE responses in vitro and in vivo, and have other putative roles in the development of allergy aside from their direct activation of IgE [27–33]. There are also considerable data supporting a role for lung and nasal eosinophils in the pathogenesis of allergic airway diseases, as well as the cytokines and other chemo-attractants that contribute to the elevated levels of eosinophils [34–36].

Abbreviations: Ab, antibody; CD23, low affinity receptor for IgE; ConA, concanavalin A; DNP, dinitrophenol; GAHE, goat anti-human IgE antibody; GAME, goat anti-mouse IgE antibody; Ig, immunoglobulin; IL, interleukin; i.p., intraperitoneal; KLH, Keyhole Limpet Hemocyanin; PBL, peripheral blood lymphocytes.

* Corresponding author. Tel.: +1 858 622 5225; fax: +1 858 658 7447.
E-mail address: mrichards@avanir.com (M.L. Richards).
[1] Present address: Neurocrine Biosciences, Inc., 12790 El Camino Real, San Diego, CA 92130, USA.

0223-5234/$ - see front matter © 2006 Elsevier SAS. All rights reserved.
doi:10.1016/j.ejmech.2006.03.014

IL-5, for example, has been shown to play a prominent role in the recruitment of eosinophils to the lung and the subsequent pulmonary pathophysiology associated with allergen exposure [37–39]. Other mediators too numerous to list have been reported to contribute to the development of allergy and asthma [13,14]. This complexity of allergic pathology impedes our efforts to establish disease control.

While there are a number of pharmacological agents available for the treatment of asthma and allergic rhinitis, a major shortcoming of many of these therapeutic alternatives is that they impact the disease state by targeting a single mediator that modifies a response at the target organ. By acting on effecter molecules, these drugs provide some symptomatic relief but do not modulate the course of the disease. Anti-histamines, for example, continue to be the drugs of choice for allergic rhinitis because they are somewhat effective and are linked to few side effects. However, anti-histamines provide little benefit for most cases of asthma, and require chronic dosing to achieve optimal effectiveness in allergic rhinitis. Leukotriene receptor antagonists have more recently been developed for the treatment of asthma but their focus on a single effecter molecule limits efficacy to a minority of patients [40].

More recently an anti-IgE antibody, omalizumab (Xolair) [15–17], has been approved by the FDA for the treatment of asthma. The clinical activity of this anti-IgE antibody has provided a sound, clinically proven basis for IgE down-regulation as a strategy for the treatment of allergy and asthma, and gilded a path for the development of orally active small molecule IgE inhibitors [41–45]. However, in spite of its central position in the expansion of allergic response, IgE is not absolutely required for disease development in animal models of asthma [46], and its suppression by the anti-IgE antibody has not been shown to reverse asthma pathology [15]. Moreover for omalizumab to be effective, serum IgE levels must be depleted by more than 95%, a level that has been unachievable by small molecule IgE inhibitors in vivo [16,41,42,45]. Other antibodies/proteins have been developed to modulate the regulation of allergic responses, including anti-IL-5 antibody [47,48], the Th1 cell response promoter IL-12 [49], soluble IL-4 receptor [50,51], and anti-CD23 antibody [52]. However, as noted above, the single protein directedness of these target-based approaches limits their effectiveness.

It is apparent that for a drug to be effective against allergic asthma, an action on a target that influences multiple mediators within the allergy cascade is ultimately required. As such, the broadly acting corticosteroids remain the cornerstone of asthma pharmacotherapy [53,54]. However, these agents are most effective when administered daily, a strategy that is associated with severe side effects and thus is reserved for serious exacerbations of the disease. Although less toxic, inhaled corticosteroids cause untoward effects with chronic use, and their effectiveness diminish with the increasing severity of asthma. Thus, there is a clear need for improvements in the treatment of moderate to severe asthma.

With the objective of filling this void in asthma pharmacotherapy we reasoned that a compound that interferes with the IgE response would act on a target that is fundamentally linked to allergy development. Implementation of this strategy through screening a proprietary compound library for activity in an ex vivo IgE assay resulted in the identification of a lead structure, and this was subsequently expanded to a library of derivatives sharing the ability to suppress IgE responses. Recently, we reported preliminary data on IgE-suppressing activity of one series of 2-(substituted phenyl) benzimidazole derivatives headlined by the lead compound AVP-13358, which is currently in clinical trials [55]. Herein, we have greatly expanded the structural characterization of the active 2-phenyl benzimidazole derivatives. Analysis of oral bioavailability has also been integrated in a detailed structure–activity relationship (SAR) in order to identify a lead compound. Additionally, we have broadened the pharmacological profile of these compounds, including a quantification of their effects on other markers of allergic disease such as CD23 and Th2 cytokines, and have shown that the inhibition of these markers of allergy are suppressed in parallel with the IgE response. Finally, we show that the 2-phenyl benzimidazole compounds suppress IgE in cultures of human peripheral blood cells, and that they reduce the IgE response in an in vivo mouse model. Their activity against a diverse group of allergy mediators thus establishes these compounds as powerful new tools for the treatment of allergy-based diseases such as asthma and allergic rhinitis.

2. Chemistry

Screening an internal universal informer library of structurally diverse compounds identified the 2-phenyl benzimidazole **1a** (Table 1) as a down regulator of IgE ex vivo (IC_{50} = 8 nM). The compound is the first in the series of 2-(substituted phenyl)-benzimidazole derivatives and formed the structural basis for the synthesis of a focused library of compounds to identify the candidates with a more suitable drug-like profile. Analogs of the benzimidazole **1a** were synthesized in four different series as shown in Schemes 1–4.

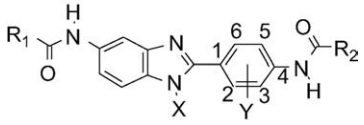
2.1. Scheme 1

The benzimidazole backbone **7** for series 1 was prepared by heating 4-nitro-phenylenediamine (**5**) and 4-amino benzoic acid (**6**) in PPA at 150 °C for 6 h. The amine **7** was then coupled with an appropriate acyl and aryl acid chloride in pyridine or THF/pyridine mixture to give **8**. The nitro-amide **8** was reduced to the amine **9** by hydrogenation with Pd–C in MeOH–THF and the resulting amine **9** was reacted with the appropriate acyl or aryl chloride to yield compounds **1a** to **1yy**.

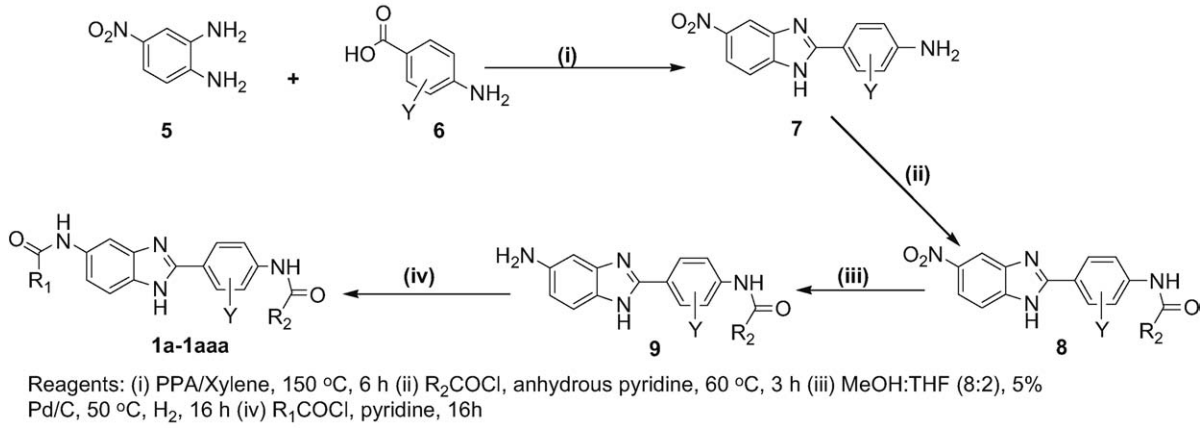
2.2. Scheme 2

Series 2 (double reverse amides of series 1) consists of compounds containing a dicarbox-diamide linked benzimidazole **12** prepared by the coupling of 3,4-diaminobenzoic acid (**10**) and 4-carbomethoxy benzaldehyde (**11**) in hot nitrobenzene for 18 h. The acid **12** was converted to the amide **13** by CDI cou-

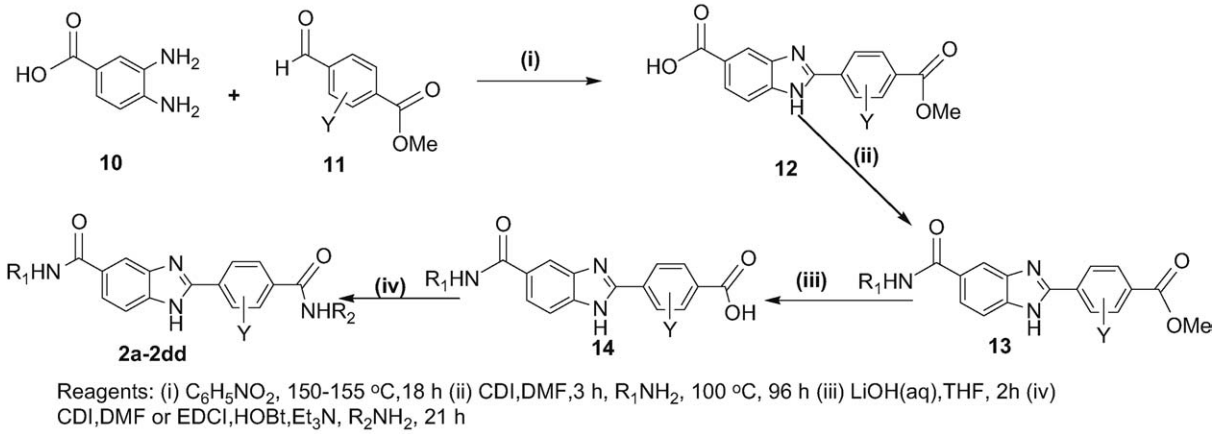
Table 1
Structure and biological activity of 2-phenyl benzimidazoles against IgE responses ex vivo and in vitro: series 1



Compounds	R1	R2	X	Y	Formula	IgE Ex vivo IC50 (nM)	IgE In vitro IC50 (nM)
1a	Phenyl	Phenyl	H	H	$C_{27}H_{20}N_4O_2$	8	20
1b	4-Bromophenyl	4-Bromophenyl	H	H	$C_{27}H_{18}Br_2N_4O_2$	20	200
1c	4-Fluorophenyl	4-Fluorophenyl	H	H	$C_{27}H_{18}F_2N_4O_2$	9	
1d	4-Chlorophenyl	4-Chlorophenyl	H	H	$C_{27}H_{18}Cl_2N_4O_2$	12	
1e	3-Chlorophenyl	3-Chlorophenyl	H	H	$C_{27}H_{18}Cl_2N_4O_2$	5	25
1f	2-Chlorophenyl	2-Chlorophenyl	H	H	$C_{27}H_{18}Cl_2N_4O_2$	5	45
1g	3,4-Dichlorophenyl	3,4-Dichlorophenyl	H	H	$C_{27}H_{16}Cl_4N_4O_2$	2	40
1h	2,3-Dichlorophenyl	2,3-Dichlorophenyl	H	H	$C_{27}H_{16}Cl_4N_4O_2$	0.7	10
1i	3,5-Dichlorophenyl	3,5-Dichlorophenyl	H	H	$C_{27}H_{16}Cl_4N_4O_2$	6	70
1j	2,4-Dichlorophenyl	2,4-Dichlorophenyl	H	H	$C_{27}H_{16}Cl_4N_4O_2$	3	30
1k	2,6-Dichlorophenyl	2,6-Dichlorophenyl	H	H	$C_{27}H_{16}Cl_4N_4O_2$	50	400
1l	4-CF3-phenyl	4-CF3-phenyl	H	H	$C_{29}H_{18}F_6N_4O_2$	2	
1m	Penta-fluoro-phenyl	Penta-fluoro-phenyl	H	H	$C_{27}H_{10}F_{10}N_4O_2$	0.5	4
1n	Phenyl	4-Chlorophenyl	H	H	$C_{27}H_{19}ClN_4O_2$	15	90
1o	4-Nitrophenyl	4-Nitrophenyl	H	H	$C_{27}H_{18}N_6O_6$	6	150
1p	4-Aminophenyl	4-Aminophenyl	H	H	$C_{27}H_{22}N_6O_2$	17	
1q	4-Cyanophenyl	4-Cyanophenyl	H	H	$C_{29}H_{18}N_6O_2$	13	100
1r	4-Methoxyphenyl	4-Methoxyphenyl	H	H	$C_{29}H_{24}N_4O_4$	4	30
1s	3,4-Dimethoxyphenyl	3,4-Dimethoxyphenyl	H	H	$C_{31}H_{28}N_4O_6$	150	700
1t	3,4,5-Trimethoxyphenyl	3,4,5-Trimethoxyphenyl	H	H	$C_{33}H_{32}N_4O_8$	> 2500	
1u	4-Methoxyphenyl	Phenyl	H	H	$C_{33}H_{32}N_4O_8$	30	
1v	4-S-methyl-phenyl	4-S-methyl-phenyl	H	H	$C_{29}H_{24}N_4O_2S_2$	80	150
1w	4-Methylphenyl	4-Methylphenyl	H	H	$C_{29}H_{24}N_4O_2$	4	20
1x	4-Phenyl-phenyl	4-Phenyl-phenyl	H	H	$C_{39}H_{28}N_4O_2$	> 2500	>2500
1y	1-Naphthalene	1-Naphthalene	H	H	$C_{35}H_{24}N_4O_2$	12	80
1z	CH_2-2-thiophene	CH_2-2-thiophene	H	H	$C_{25}H_{20}N_4O_2S_2$	55	500
1aa	Cyclohex-3-ene	Cyclohex-3-ene	H	H	$C_{27}H_{28}N_4O_2$	2	40
1bb	CH_3	CH_3	H	H	$C_{17}H_{16}N_4O_2$	> 2500	>2500
1cc	Phenyl-	Cyclohexyl	H	H	$C_{27}H_{26}N_4O_2$	7	10
1dd	CH_3	Cyclohexyl	H	H	$C_{22}H_{24}N_4O_2$	50	100
1ee	3,4-Dichlorophenyl	Cyclohexyl	H	H	$C_{27}H_{24}Cl_2N_4O_2$	0.8	0.8
1ff	4-Chlorophenyl	Cyclohexyl	H	H	$C_{27}H_{25}ClN_4O_2$	1	6
1gg	Cyclohexyl	3,4-Dichlorophenyl	H	H	$C_{27}H_{24}Cl_2N_4O_2$	0.8	0.4
1hh	Cyclohexyl	4-Chlorophenyl	H	H	$C_{27}H_{25}ClN_4O_2$	4	8
1ii	1-Adamantyl	2-Fluorophenyl	H	H	$C_{31}H_{29}FN_4O_2$		10
1jj	1-Adamantyl	4-Fluorophenyl	H	H	$C_{31}H_{29}FN_4O_2$		10
1kk	2-Pyridyl	1-Adamantyl	H	H	$C_{30}H_{29}N_5O_2$		6
1ll	3-Pyridyl	1-Adamantyl	H	H	$C_{30}H_{29}N_5O_2$		20
1mm	Cyclohexyl	Cyclohexyl	H	H	$C_{27}H_{32}N_4O_2$	1	4
1nn	1-Adamantyl	1-Adamantyl	H	H	$C_{35}H_{40}N_4O_2$	1	4
1oo	Cycloheptyl	Cycloheptyl	H	H	$C_{29}H_{36}N_4O_2$	0.5	1.5
1pp	Cyclopentyl	Cyclopentyl	H	H	$C_{25}H_{28}N_4O_2$	4	
1qq	Cyclobutyl	Cyclobutyl	H	H	$C_{23}H_{24}N_4O_2$	80	400
1rr	Cyclopropyl	Cyclopropyl	H	H	$C_{21}H_{20}N_4O_2$	700	1000
1ss	4-Methyl-cyclohexyl	4-Methyl-cyclohexyl	H	H	$C_{29}H_{36}N_4O_2$	1	4
1tt	2-Methyl-cyclohexyl	2-Methyl-cyclohexyl	H	H	$C_{29}H_{36}N_4O_2$	0.5	
1uu	2-Pyrrolidine	2-Pyrrolidine	H	H	$C_{23}H_{26}N_6O_2$	> 2000	
1vv	Cinnamyl	Cinnamyl	H	H	$C_{31}H_{24}N_4O_2$	12	70
1ww	Phenyl	Phenyl	n-Butyl	H	$C_{31}H_{28}N_4O_2$	2000	
1xx	Phenyl	Phenyl	CH_3	H	$C_{28}H_{22}N_4O_2$	500	800
1yy	Cyclohexyl	Cyclohexyl	$COOCH_2CH_3$	H	$C_{30}H_{36}N_4O_4$	3	7
1zz	Cyclohexyl	Cyclohexyl	$COCH_3$	H	$C_{29}H_{34}N_4O_3$	1	1.5
1aaa	Cyclohexyl	Cyclohexyl	H	2-F	$C_{27}H_{31}FN_4O_2$		2



Scheme 1. Synthesis of 2-substituted phenylbenzimidazole series 1.



Scheme 2. Synthesis of 2-substituted phenylbenzimidazole series 2.

pling with amines. The ester-amide **13** was hydrolyzed with aq. LiOH in THF to afford the corresponding acid **14**, which was appended to the appropriate amine using standard CDI or EDCI/HOBt/Et_3N coupling to afford the amides **2a**–**2dd**.

2.3. Scheme 3

In series 3 (mono reverse amide of series 2) the nitrocarbomethoxy-benzimidazole **15** was prepared by heating 4-nitophenylenediamine (**5**) and 4-carbomethoxy benzaldehyde (**11**) in nitrobenzene at 155–160 °C for 18 h. The ester group of **15** was saponified with aq. LiOH in THF to the acid **16**, which was eventually converted to a nitro-amide **17** by CDI coupling to an amine. The nitro-amide **17** was reduced to the amine **18** and coupled with the appropriate acid chlorides to afford the amides **3a**–**3aa**.

2.4. Scheme 4

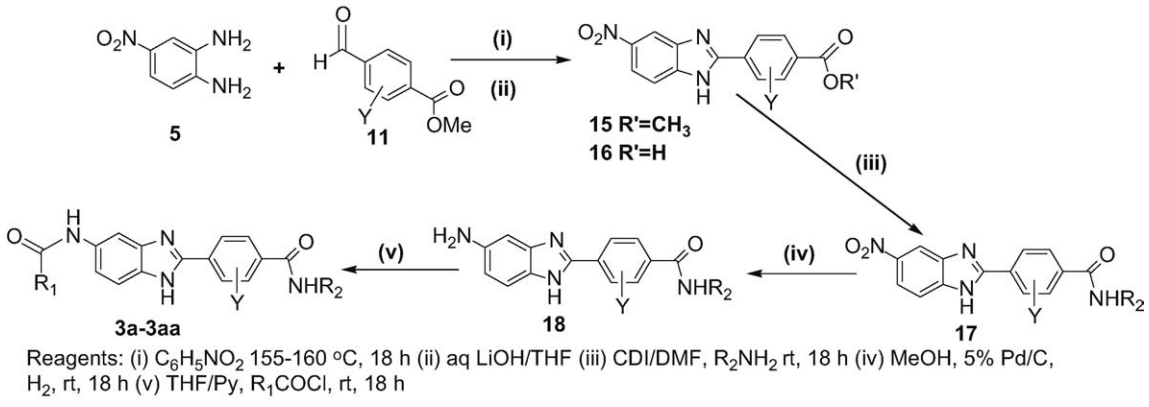
In series 4 (mono reverse amide of series 1), the nitro-acid benzimidazole core **20** was prepared by condensing 3,4-diaminobenzoic acid (**10**) with 4-nitrobenzaldehyde (**19**) in nitrobenzene at 155–160 °C for 18 h. The nitro acid **20** was converted to an amide **21**, by CDI or EDCI coupling to an amine. The nitro-amide **21** was reduced to the amine **22**, and finally coupled with the appropriate acid chlorides to afford the amides **4a**–**4nnn**.

3. Results and discussion

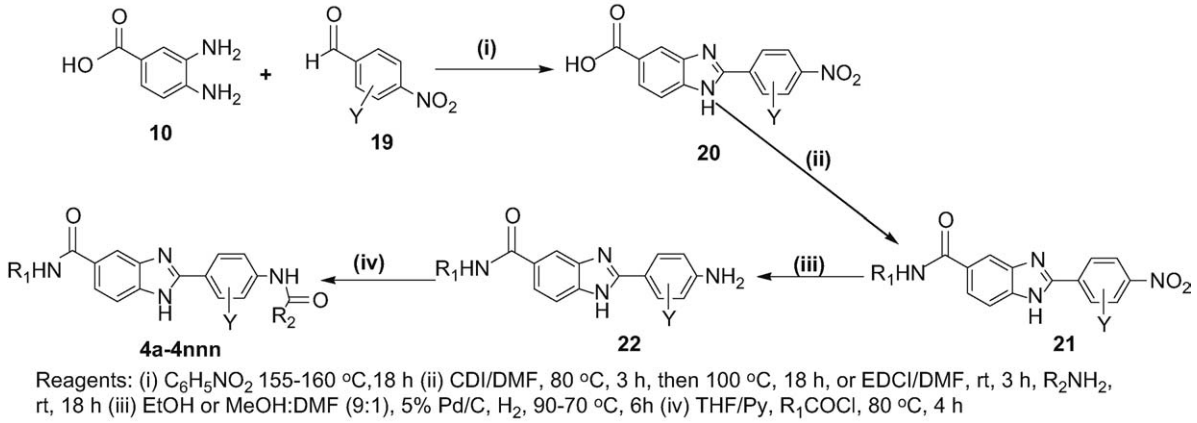
3.1. Ex vivo/in vitro IgE response

IgE is centrally positioned in the allergy cascade, and is regulated by molecules that play a prominent role in allergy such as IL-4, IL-13, and various co-stimulating molecules within the Th2 response system. We reasoned that a compound that inhibits IgE response would potentially interfere with a molecule that is fundamental to the development or perpetuation of allergic responses. Thus, a cell-based assay was used to identify inhibitors of antigen-specific IgE response in vitro. Although it contributes little understanding of the pharmacological action, a cell-based approach is far more likely to identify agents that act on unexplored response pathways than target-based screening.

The particular ex vivo IgE response assay was selected for compound screening because of its capacity to reveal activity against allergen responses in either B or T lymphocytes. The assay utilizes in vivo priming with antigen followed by in vitro



Scheme 3. Synthesis of 2-substituted phenylbenzimidazole series 3.



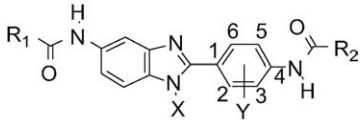
Scheme 4. Synthesis of 2-substituted phenylbenzimidazole series 4.

antigen-specific IgE response in the presence of test compounds [56]. Although IgE is the readout of the assay, its inhibition can result from an action on any of the processes known to be important for the amplification of atopic diseases. Thus, it can expose an agent with activity against any of a number of processes in the secondary immune response including antigen presentation, expression of co-stimulatory molecules or cytokine receptors, or synthesis/release of IL-4, IL-13, or IgE.

The ex vivo IgE response assay was used to screen an internal universal informer library that was generated from over 300,000 compounds. Of the compound hits generated from the initial screen, a cluster of chemically similar compounds containing the 2-(substituted phenyl)-benzimidazole core structure showed moderate potency for inhibiting IgE. The most potent compound identified during this discovery phase is **1a**, which led to the synthesis of derivatives containing the core 2-phenyl benzimidazole scaffold. This focused synthetic effort resulted in the generation of compounds with IC_{50}s in the ex vivo IgE response assay that range from 400 pM to over 3 μM (Tables 1–4).

IgE secretion by B cell-derived lymphocytes requires either IL-4 or IL-13 and the engagement of CD40 on their cell surface. In the ex vivo IgE response assay, these components are provided by spleen T cells that were primed in vivo to respond to specific antigen (DNP-KLH) [56]. Culturing B and T cells from spleens of sensitized mice in the presence of DNP-KLH during the in vitro portion of the protocol thus provides all the components necessary for generating IgE. A similar response can also be achieved by isolating B cells from mouse spleen and culturing them with recombinant murine IL-4 and anti-CD40 antibody (in vitro IgE response assay), thus negating the need for T cell help in generating the IgE response. The in vitro assay with isolated mouse splenic B cells thus directly measures the B cell response to stimulus. Because IL-4 is supplied exogenously, compounds such as suplatast tosilate that inhibit IgE via inhibition of Th2 cytokines would not inhibit IgE in the in vitro assay [41–45]. As shown in Tables 1–4, all 2-phenyl benzimidazole compounds that are active in the ex vivo IgE response assay also suppress IgE in the in vitro assay, although two to fivefold higher concentrations are typically required in the latter. Thus, while the results in the IL-4/anti-CD40 antibody (Ab) system indicate that the 2-(substituted phenyl)-benzimidazole derivatives act directly on B cells, the lower potency of these compounds in the in vitro assay relative to the ex vivo assay suggests that other components (e.g. T cells) of the IgE response are affected. Although initial development of the SAR was based on activity in both the ex vivo and in vitro IgE assays, the latter was utilized in subsequent analyses because a) the results in the two assays follow

Table 2
Structure and biological activity of 2-phenyl benzimidazoles against IgE responses ex vivo and in vitro: series 2



Compounds	R1	R2	Y	Formula	IgE Ex vivo IC50 (nM)	IgE In vitro IC50 (nM)
2a	4-Methyl-phenyl	4-Methyl-phenyl	H	$C_{29}H_{24}N_4O_2$	55	40
2b	4-Fluorophenyl	4-Fluorophenyl	H	$C_{27}H_{18}F_2N_4O_2$	200	150
2c	4-Methoxyphenyl	4-Methoxyphenyl	H	$C_{29}H_{24}N_4O_4$	40	100
2d	Phenyl	3,4-Dichlorophenyl	H	$C_{27}H_{18}Cl_2N_4O_2$		100
2e	Phenyl	5-Methyl-2-pyridyl	H	$C_{27}H_{21}N_5O_2$		300
2f	Cyclohexyl	Phenyl	H	$C_{27}H_{26}N_4O_2$		9
2g	1-Adamantyl	Phenyl	H	$C_{31}H_{30}N_4O_2$		25
2h	Phenyl	1-Adamantyl	H	$C_{31}H_{30}N_4O_2$		8
2i	1-Adamantyl	4-Chlorophenyl	H	$C_{31}H_{29}ClN_4O_2$		9
2j	1-Adamantyl	3,4-Dichlorophenyl	H	$C_{31}H_{28}Cl_2N_4O_2$	0.8	1.5
2k	2-Adamantyl	3,4-Dichlorophenyl	H	$C_{31}H_{28}Cl_2N_4O_2$		16
2l	Cyclohexyl	4-Fluorophenyl	H	$C_{27}H_{25}FN_4O_2$		5
2m	Cyclohexyl	4-Chlorophenyl	H	$C_{27}H_{25}ClN_4O_2$		3
2n	2-Adamantyl	4-Methoxyphenyl	H	$C_{32}H_{32}N_4O_3$		7
2o	4-Methoxyphenyl	1-Adamantyl	H	$C_{32}H_{32}N_4O_3$		40
2p	4-Fluorophenyl	2-Adamantyl	H	$C_{31}H_{29}FN_4O_2$		40
2q	1-Adamantyl	2-Pyridyl	H	$C_{30}H_{29}N_5O_2$		10
2r	2-Adamantyl	2-Pyridyl	H	$C_{30}H_{29}N_5O_2$		10
2s	2-Adamantyl	3-Pyridyl	H	$C_{30}H_{29}N_5O_2$		20
2t	2-Adamantyl	4-Pyridyl	H	$C_{30}H_{29}N_5O_2$		40
2u	2-Pyridyl	2-Adamantyl	H	$C_{30}H_{29}N_5O_2$		40
2v	2-Pyridyl	1-Adamantyl	H	$C_{30}H_{29}N_5O_2$	10	40
2w	2-Adamantyl	5-Methyl-2-pyridyl	H	$C_{31}H_{31}N_5O_2$		20
2x	Cyclohexyl	Cyclohexyl	H	$C_{27}H_{32}N_4O_2$	35	80
2y	1-Adamantyl	1-Adamantyl	H	$C_{35}H_{40}N_4O_2$	20	16
2z	4-Methyl-cyclohexyl	4-Methyl-cyclohexyl	H	$C_{29}H_{36}N_4O_2$	10	35
2aa	Cyclohexyl	1-Adamantyl	H	$C_{31}H_{36}N_4O_2$	15	8
2bb	1-Adamantyl	Cyclohexyl	H	$C_{31}H_{36}N_4O_2$		35
2cc	2-Adamantyl	2-Methyl.cyclohexyl	H	$C_{32}H_{38}N_4O_2$		4
2dd	2-Methyl-cyclohexyl	1-Adamantyl	H	$C_{32}H_{38}N_4O_2$		4

in parallel, b) the ex vivo assay is complicated by the requirement of multiple cell types, and c) the in vitro system is more robust, quicker, and less labor-intensive.

3.2. Oral bioavailability

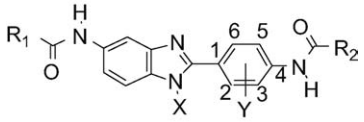
In addition to showing potent activity in an appropriate biological assay, an allergy drug must achieve adequate serum levels following oral administration. This requirement was a critical factor in evaluating candidate compounds for further development. Oral bioavailability was assessed by administering 40 mg/kg of compound suspended at 10 mg/ml in 0.2 M citric acid/PBS (pH 4) by oral gavage. Serum samples were obtained at 1 and 4 h after dosing and quantified by comparison of HPLC tracings from serum samples to a standard curve. Compounds having active metabolites (determined by measuring bioactivity of HPLC eluates) were evaluated on the basis of total active compound in the serum. Overall, oral bioavailability was low for all four benzimidazole series, although series 4 compounds achieved the highest serum concentrations of those tested (Table 4). The data for selected compounds and their comparison with other series are shown in Table 5. Oral bioavailability as a guide for choosing the lead compound is discussed in detail in Section 3.4.

3.3. Structure–activity relationships

The SARs of the 2-(substituted phenyl)-benzimidazole compounds were established through analyses of four series of compounds that differ in the orientation of the bis-amide linkages. The synthetic focus was also influenced by the relative oral bioavailability, as discussed in next section.

The active 2-(substituted phenyl)-benzimidazole (2-PB) moiety was initially identified based on the activity of **1a**. This compound contains the 2-PB backbone flanked by amide-linked phenyl groups, and has moderate potency for suppressing IgE response (IC50 = 20 nM in vitro; Table 1). The 2-phenyl benzimidazole backbone showed little tolerance for change as a substantial loss of activity resulted from modifications to the core structure; including replacement of the carbonyl or the amine of the amide linkages with methyls, modification of the benzimidazole or 2-phenyl groups, substitution of a

Table 3
Structure and biological activity of 2-phenyl benzimidazoles against IgE responses ex vivo and in vitro: series 3



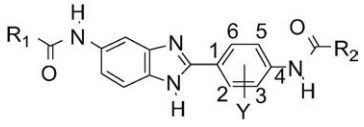
Compounds	R1	R2	Y	Formula	IgE Ex vivo IC50 (nM)	IgE In vitro IC50 (nM)
3a	Phenyl	Phenyl	H	$C_{22}H_{24}N_4O_2$	200	400
3b	3,4-Dichlorophenyl	Phenyl	H	$C_{27}H_{18}Cl_2N_4O_2$	30	50
3c	Phenyl	Cyclohexyl	H	$C_{27}H_{26}N_4O_2$	35	70
3d	Cyclohexyl	Phenyl	H	$C_{27}H_{26}N_4O_2$	70	130
3e	1-Adamantyl	Phenyl	H	$C_{31}H_{30}N_4O_2$	30	150
3f	Phenyl	1-Adamantyl	H	$C_{31}H_{30}N_4O_2$	1.5	4
3g	3,4-Dichlorophenyl	1-Adamantyl	H	$C_{31}H_{28}Cl_2N_4O_2$	1	0.7
3h	3,4-Dichlorophenyl	Bicycloheptyl	H	$C_{28}H_{24}Cl_2N_4O_2$	20	40
3i	3,4-Dichlorophenyl	Cyclohexyl	H	$C_{27}H_{24}Cl_2N_4O_2$	7	15
3j	Cyclohexyl-	CH_3	H	$C_{22}H_{24}N_4O_2$	1000	> 2000
3k	CH_3	CH_3	H	$C_{17}H_{16}N_4O_2$	> 2000	> 2000
3l	CH_3	Cyclohexyl	H	$C_{22}H_{24}N_4O_2$	300	> 2000
3m	Cyclohexyl	Cyclohexyl	H	$C_{27}H_{32}N_4O_2$	15	50
3n	1-Adamantyl	1-Adamantyl	H	$C_{26}H_{28}N_4O_2$	3	6
3o	Cycloheptyl	Cycloheptyl	H	$C_{29}H_{36}N_4O_2$	2	3
3p	Cyclopentyl	Cyclopentyl	H	$C_{25}H_{28}N_4O_2$	220	500
3q	Bicycloheptyl	Bicycloheptyl	H	$C_{29}H_{32}N_4O_2$	70	30
3r	2-Methyl-cyclohexyl	2-Methyl-cyclohexyl	H	$C_{29}H_{36}N_4O_2$	9	60
3s	4-Methyl-cyclohexyl	4-Methyl-cyclohexyl	H	$C_{29}H_{36}N_4O_2$	6	3
3t	1-Adamantyl	Cyclohexyl	H	$C_{31}H_{36}N_4O_2$	8	60
3u	Cyclohexyl	1-Adamantyl	H	$C_{31}H_{36}N_4O_2$	1	4
3v	4-Methyl-cyclohexyl	1-Adamantyl	H	$C_{32}H_{38}N_4O_2$	1	3
3w	Cyclohexyl	Bicycloheptyl	H	$C_{28}H_{32}N_4O_2$	25	30
3x	1-Adamantyl	Bicycloheptyl	H	$C_{23}H_{24}N_4O_2$	60	20
3y	1-Adamantyl	Cycloheptyl	H	$C_{32}H_{38}N_4O_2$	7	3
3z	1-Adamantyl	Cyclopentyl	H	$C_{30}H_{34}N_4O_2$	70	70
3aa	2-Pyridyl	1-Adamantyl	H	$C_{30}H_{29}N_5O_2$		40

benzthiazole for the benzimidazole, replacement of the amide linkages with sulfonamides, substitution on the imidazole nitrogen, and replacement of amide linkages with terminal halogens, amines, nitros, or methyls. The following SAR analyses thus focuses primarily on changes in both the amide orientation and terminal group substitutions.

Initial attempts to improve the potency of this series by 4-halo substitutions on the phenyl rings at R_1 and R_2 generally resulted in equivalent or less potent derivatives (**1b**, **1c**, **1d**). Substitution at the 4-position prefers the order of F > Cl > Br. Chloro-substitutions at the 2 or 3 position (**1e**, **1f**) either retained or improved the potency over the 4-position (**1d**). Bis-halo substitutions generally provided higher potency over single halogens, and chloro-substitution at the 2,3 positions (**1h**) is preferred to the 3,4 (**1g**), the 3,5 (**1i**), or the 2,4 (**1j**) positions, all of which are preferred to the 2,6 position (**1k**). Addition of electro-negative groups, either penta-fluoro or *p*-CF_3, also improved the potency over the unsubstituted aryls (**1l**, **1m**), which together with the 2,3-dichloro compound (**1h**) were the most potent of the bis-aryls. Introduction of an electron donating group such as methoxyl or methyl at the 4-position of the aromatic at R_1 and R_2 produced compounds with no improvement in potency (**1r**, **1w**) whereas substitution with two or more methoxy groups resulted in much less active compounds (**1s**, **1t**). Compounds with terminal phenyl groups substituted with nitro (**1o**), S-methyl (**1v**), amino (**1p**), or cyano (**1q**) also were less potent than the parent (**1a**). Methyl substitution on the terminal aryl (**1w**) had little effect, but the addition of bulky aryls such as a *p*-phenyl (**1x**) resulted in a loss of activity. The bis-naphthalene derivative (**1y**), however, was nearly as potent as bis-phenyl compound (**1a**). Replacement of aromatic groups with methyl-thiophenes (**1z**) or with methyls at one or both R positions (**1bb**, **1dd**) resulted in a substantial loss of activity indicating the importance of bulky hydrophobic groups for IgE suppression.

Replacement of terminal aryls with cycloalkyl groups generally improved the potency of series 1 benzimidazoles. Substitution of a cycloalkyl in place of one of the terminal aryls resulted in approximately equivalent activity as the bis-aryl (**1a** vs. **1cc**). However, when halogen-substituted aryls are used together with terminal cycloalkyls at R_1 or R_2 (**1ee**, **1ff**, **1gg**), potency was increased about 10-fold. Large bis-cycloalkyls (**1mm**, **1nn**, **1oo**) or hetero-aryl in combination with a fused cycloalkyl group (**1kk**) also provided higher potency than bis aryls. Methyl substitutions on the cyclohexyl (**1ss**, **1tt**) did not substantially improve potency over the unsubstituted com-

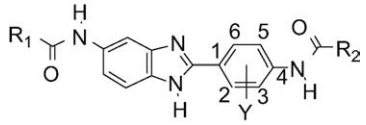
Table 4
Structure and biological activity of 2-phenyl benzimidazoles against IgE responses ex vivo and in vitro: series 4



Compounds	R1	R2	Y	Formula	IgE Ex vivo IC50 (nM)	IgE In vitro IC50 (nM)	Serum BA[a]
4a	Phenyl	Phenyl	H	$C_{27}H_{20}N_4O_2$	120	200	
4b	2-Pyridyl	Phenyl	H	$C_{26}H_{19}N_5O_2$	100	350	800
4c	2-Pyridyl	2-OCF3-phenyl	H	$C_{27}H_{18}F_3N_5O_3$		250	9240[b]
4d	2-Pyridyl	3,4-Dichlorophenyl	H	$C_{26}H_{17}Cl_2N_5O_2$	90	60	
4e	1-Adamantyl	Phenyl	H	$C_{31}H_{30}N_4O_2$	1.5	6	
4f	Phenyl	1-Adamantyl	H	$C_{31}H_{30}N_4O_2$	20	60	
4g	1-Adamantyl	4-Chlorophenyl	H	$C_{31}H_{29}ClN_4O_2$	0.5	0.8	
4h	1-Adamantyl	4-Nitrophenyl	H	$C_{31}H_{29}N_5O_4$	5	6	
4i	1-Adamantyl	3,4-Dichlorophenyl	H	$C_{31}H_{28}Cl_2N_4O_2$	0.4	2	
4j	1-Adamantyl	Penta-fluoro-benzene	H	$C_{31}H_{25}F_5N_4O_2$	2	5	
4k	Cyclohexyl	Phenyl	H	$C_{27}H_{26}N_4O_2$	8	24	
4l	Cyclohexyl	3,4-Dichlorophenyl	H	$C_{27}H_{24}Cl_2N_4O_2$	5	7	
4m	Cyclohexyl	4-Chlorophenyl	H	$C_{27}H_{25}ClN_4O_2$	7	6	
4n	Bicycloheptyl	Phenyl	H	$C_{28}H_{26}N_4O_2$	50	50	
4o	Phenyl	Cyclohexyl	H	$C_{27}H_{26}N_4O_2$	40	60	
4p	4-Azido-phenyl	1-Adamantyl	H	$C_{31}H_{29}N_7O_2$		7	7
4q	4-Amino-phenyl	1-Adamantyl	H	$C_{31}H_{31}N_5O_2$		100	
4r	1-Adamantyl	2-Fluorophenyl	H	$C_{31}H_{29}FN_4O_2$		10	250*
4s	Cyclohexyl	2-Fluorophenyl	H	$C_{27}H_{25}FN_4O_2$		40	425
4t	Cyclohexyl	2-OCF3-phenyl	H	$C_{28}H_{25}F_3N_4O_3$		40	750
4u	Cyclohexyl	4-OCF3-phenyl	H	$C_{28}H_{25}F_3N_4O_3$		10	23
4v	1-Adamantyl	2-OCF3-phenyl	H	$C_{32}H_{29}F_3N_4O_3$		10	355*
4w	2-Fluorophenyl	Cyclohexyl	H	$C_{27}H_{25}FN_4O_2$		40	450
4x	4-Fluorophenyl	Cyclohexyl	H	$C_{27}H_{25}FN_4O_2$		10	100
4y	1-Adamantyl	2-Pyridyl	H	$C_{30}H_{29}N_5O_2$	3	7	20
4z	2-Pyridyl	1-Adamantyl	H	$C_{30}H_{29}N_5O_2$	5	8	600[b]
4aa	3-Pyridyl	1-Adamantyl	H	$C_{30}H_{29}N_5O_2$	20	35	150
4bb	4-Pyridyl	1-Adamantyl	H	$C_{30}H_{29}N_5O_2$	35	35	100
4cc	2-Pyridyl	Cyclohexyl	H	$C_{26}H_{25}N_5O_2$	25	50	
4dd	2-Pyridyl	3-Acetoxy-1-adamantyl	H	$C_{32}H_{31}N_5O_4$		3	155[b]
4ee	2-Pyridyl	4-Keto-1-adamantyl	H	$C_{30}H_{29}N_5O_3$	10	10	155[b]
4ff	2-Pyridyl	Bicycloheptyl	H	$C_{27}H_{25}N_5O_2$	80	60	
4gg	2-Pyridyl	3-OH-1-adamantyl	H	$C_{30}H_{29}N_5O_3$		90	25
4hh	2-Pyridyl	2-OH-1-adamantyl	H	$C_{30}H_{29}N_5O_3$		80	
4ii	2-Pyridyl	4-OH-1-adamantyl	H	$C_{30}H_{29}N_5O_3$		5	40
4jj	2-Pyridyl	4-O-methyloxime-1-adamantyl	H	$C_{31}H_{30}N_6O_3$		30	1500
4kk	2-Pyridyl	4-F-1-adamantyl	H	$C_{30}H_{28}FN_5O_2$		2	230
4ll	2-Pyridyl	3-Br-1-adamantyl	H	$C_{30}H_{28}BrN_5O_2$		2	15
4mm	4,6-Dimethyl-2-pyridyl	1-Adamantyl	H	$C_{32}H_{33}N_5O_2$		3	
4nn	2-Pyridyl	1-Methyl-adamantyl	H	$C_{31}H_{31}N_5O_2$		40	7
4oo	H	1-Adamantyl	H	$C_{25}H_{26}N_4O_2$	60	220	
4pp	1-Adamantyl	Ch3	H	$C_{26}H_{28}N_4O_2$	20	90	10
4qq	Cyclohexyl	Ch3	H	$C_{22}H_{24}N_4O_2$	350	>2000	
4rr	Cyclohexyl	Cyclohexyl	H	$C_{27}H_{32}N_4O_2$	10	9	
4ss	Cyclopentyl	Cyclopentyl	H	$C_{25}H_{28}N_4O_2$	120	80	
4tt	Cycloheptyl	Cycloheptyl	H	$C_{29}H_{36}N_4O_2$	1	3	
4uu	1-Adamantyl	1-Adamantyl	H	$C_{35}H_{40}N_4O_2$	2	5	
4vv	1-Adamantyl	Bicycloheptane	H	$C_{32}H_{36}N_4O_2$	4	6	
4ww	1-Adamantyl	Cyclohexyl	H	$C_{31}H_{36}N_4O_2$	0.5	5	
4xx	2-Adamantyl	Cyclohexyl	H	$C_{31}H_{36}N_4O_2$	0.4	0.4	
4yy	Cyclohexyl	T-butyl	H	$C_{25}H_{30}N_4O_2$	55	70	
4zz	Cyclohexyl	1-Adamantyl	H	$C_{31}H_{36}N_4O_2$	3	2	
4aaa	Cyclohexyl	Bicycloheptyl	H	$C_{28}H_{32}N_4O_2$	3	10	
4bbb	Bicycloheptyl	Cyclohexyl	H	$C_{28}H_{32}N_4O_2$	4	65	

(continued)

Table 4 *(continued)*



Compounds	R1	R2	Y	Formula	IgE Ex vivo IC50 (nM)	IgE In vitro IC50 (nM)	Serum BA[a]
4ccc	2-Pyridyl	1-Adamantyl	2-OMe	$C_{31}H_{31}N_5O_3$	20	80	205[b]
4ddd	2-Pyridyl	1-Adamantyl	2-(1-Adamantane carboxylate)	$C_{41}H_{43}N_5O_4$	6	8	
4eee	2-Pyridyl	1-Adamantyl	2-OH	$C_{30}H_{29}N_5O_3$	4	8	
4fff	2-Pyridyl	1-Adamantyl	2-F	$C_{30}H_{28}FN_5O_2$	6	10	1100[b]
4ggg	2-Pyridyl	1-Adamantyl	3-F	$C_{30}H_{28}FN_5O_2$	6	35	900[b]
4hhh	2-Pyridyl	1-Adamantyl	2-CF3	$C_{31}H_{28}F_3N_5O_2$	650	1000	380[b]
4iii	2-Pyridyl	1-Adamantyl	3-OH	$C_{30}H_{29}N_5O_3$	6	12	<5
4jjj	2-Pyridyl	3-OH-1-adamantyl	3-OH	$C_{30}H_{29}N_5O_4$		1	<5
4kkk	2-Pyridyl	3-Acetoxy-1-adamantyl	3-OH	$C_{32}H_{31}N_5O_5$		1	10
4lll	2-Pyridyl	4-Keto-1-adamantyl	2-F	$C_{30}H_{26}FN_5O_3$		5	160[b]
4mmm	2-Pyridyl	4-OH-1-adamantyl	2-F	$C_{30}H_{28}FN_5O_3$		3	
4nnn	2-Pyridyl	3-OH-1-adamantyl	2-F	$C_{30}H_{28}FN_5O_3$		60	

[a] Bioavailability: ng/ml of compound in serum 1 h after a 40 mg/kg oral dose.
[b] Includes parent compound and active metabolite.

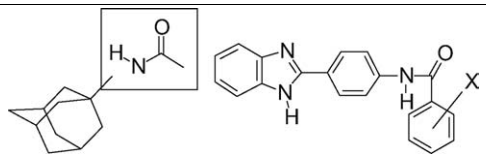
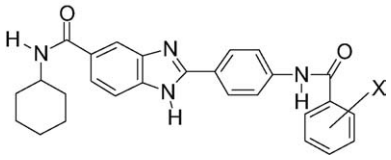
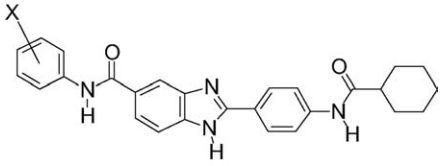
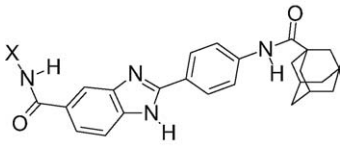
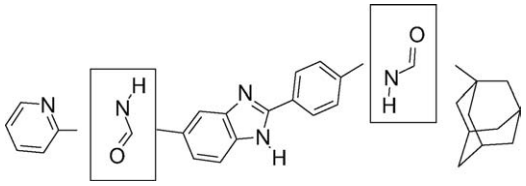
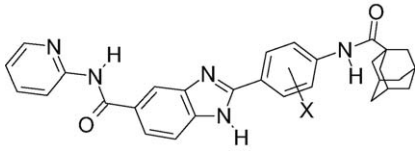
pound (**1mm**). The potency of compounds substituted with smaller cycloalkyls diminished in proportion to their size; cyclopentyl (**1pp**) is much more potent than cyclobutyl (**1qq**) or cylcopropyl (**1rr**). This further underscores the importance of bulky hydrophobic groups for IgE suppression.

As pointed out above, attempts to make substitutions on the benzimidazole backbone generally resulted in loss of activity. Modest changes such as the introduction of halogens at the 2-position of phenyl ring Y retained compound activity (**1aaa**), but substitutions on the imidazole hydrogen with aliphatic alkyl groups substantially reduced the activity (**1ww**, **1xx**). Carbamate (**1yy**) and amide derivatives (**1zz**), on the other hand, retained the potency most likely because they behave as prodrugs by hydrolyzing to the parent compound under the assay conditions. Thus, series 1 compounds require an intact 2-phenyl benzimidazole backbone and prefer large terminal hydrophobic substitutions, either cycloalkyl or aryl.

Series 2 compounds are the double reverse amides of series 1 2-phenyl benzimidazoles. Examination of the biological activity of these compounds indicates that, with few exceptions, series 2 compounds are significantly less potent than their congeners in series 1 (Table 2). This is particularly true when comparing methyl-, halo-, or methoxy-substituted aromatics (**2a**, **2b**, **2c**), or unsubstituted or methyl-substituted bis-cycloalkyls (**2v**, **2x**, **2y**, **2z**) in congeners of both series, wherein potency strongly favors series 1 compounds. However, the cyclohexyl/4-fluoro-phenyl combination at R_1 and R_2 (**2l**) show equivalent potency in series 1 and 2. A similar compound **2j** which combines 1-adamantyl with 3,4 dichloro phenyl at the termini is as potent as any compound in series 1. Moreover, insertion of other aliphatics and either halogen- or methoxy-substituted aromatics at R_1 and R_2, respectively, also results in reasonably potent compounds (**2k**, **2l**, **2m**). Compounds with similar terminal substitutions but in the reverse orientation are overall less potent (**2o**, **2p**). 2-Pyridines are more potent than 3- or 4-pyridines (**2r**, **2s**, **2t**) or the 5-methyl substituted 2-pyridine (**2w**) when paired with 2-adamantyl at R_1. With a pyridine at R_1 and adamantyl at R_2, however, potency decreases (**2v**, **2u**). Substitution of 4-methoxy phenyl at R_1 and R_2 (**2c**) or just R_1 (**2o**) gave less potent compounds than 2-adamantyl/4-methoxy-phenyl at R_1/R_2 (**2n**). As noted above for series 1, halo-substituted phenyls combined with large cycloalkyl groups at R_1 (**2i**, **2j**, **2k**, **2l**, **2m**), and mixtures of large unsubstituted or methyl-substituted cycloalkyls (**2aa**, **2cc**, **2dd**) also resulted in potent compounds. This series of benzimidazoles thus show a preference for large cycloalkyls in R_1 with halo-substituted aromatics at R_2.

Series 3 compounds were synthesized and tested to assess the functional role of mixing the amide linkage orientation. As with series 2, however, these compounds were found to be less potent than their congeners in series 1 (Table 3). This applies to bis aryl, bis alkyl, and combinations of cycloalkyls with aromatics (**3a**, **3c**, **3m**, **3p**). As the size of the aliphatic groups increase, however, the discrepancy in potency between the series diminishes (**3o**, **3n**, **3s**). Direct comparison of series 3 with series 2 compounds gave variable results. Cylcoalkyls at R_1 with phenyl at R_2 favor series 2 compounds (**2f** vs. **3d**), while the reverse orientation favors series 3 (**3f**). Bis-cycloalkyls also favor series 3 (**3m**, **3n**) while the potency of mixed cycloalkyls is similar in both series (**3t**, **3u**). When bulky aliphatic groups were inserted at R_2 with aromatic groups at R_1 (**3f**), potency of series 3 compounds increased to a level that was comparable to the most potent compounds of other series. A highly potent compound also resulted when bis-halogenated aromatic groups were used at R_1 with a bulky aliphatic group at R_2 (**3g**). Likewise, larger bis-cycloalkyl groups were preferred over smaller ones (**3m**, **3n**, **3o**, **3p**) although bicycloheptyls (**3h**, **3q**, **3w**, **3z**) were less potent than other bulky cycloalkyls. As with the

Table 5
Selected oral bioavailability comparisons of 2-phenyl benzimidazoles

	Compounds	Core Structure	X	Amide linkage	IC50	[Serum] (ng/ml)*	Bio-activity#
I	**4r**		o-F	Series 4	10	250	25
	1ii		o-F	Series 1	10	155	16
	1jj		p-F	Series 1	10	153	15
	4v		o-OCF3	Series 4	10	355	36
II	**4s**		o-F	Series 4	40	425	11
	4t		o-OCF3	Series 4	40	750	19
	4u		p-OCF3	Series 4	10	23	2
III	**4w**		o-F	Series 4	40	450	11
	4x		p-F	Series 4	10	100	10
IV	**4z**		2-pyridine	Series 4	8	600	75
	4aa		3-pyridine	Series 4	35	150	4
	4bb		4-pyridine	Series 4	35	100	3
V	**1kk**			Series 1	6	100	16
	2v			Series 2	10	410	41
	3aa			Series 3	40	450	11
	4z			Series 4	8	600	75
VI	**4z**		H	Series 4	8	600	75
	4fff		2-F	Series 4	10	1100	110
	4ggg		3-F	Series 4	35	900	26

* Serum obtained at 1 h after a 40 mg/kg dose to five female BALB/c mice (per drug). The bleeds were pooled prior to processing and quantifying by HPLC.
# Bioactivity = peak serum concentration (nM) divided by the in vitro IC50.

other series, replacement of aryls or cyclo-alkyls with small aliphatic groups at the termini of series 3 compounds (**3j**, **3k**, **3l**), drastically reduced their potency ($IC_{50} > 200$–2000 nM). Thus the mixed amide linkage orientation of series 3 compounds optimally favors aryls or large cyloalkyls at R_1 with bulky cyloalkyls at R_2 (**3s**, **3u**, **3v**, **3y**, **3n**, **3o**, **3f**, **3g**, **3i**).

Series 4 compounds, while not the most potent series, provided the required elements of a drug in that they achieve higher bioavailability than congeners in other series while retaining reasonable pharmacological potency. This series of compounds developed a similar SAR as observed with the other series (Table 4) but the low potency of bis-aryl compounds in this series (e.g. **4a**) shifted the emphasis to compounds with alkyl substitutions. Acyl and aryl combinations at R_1 and R_2 provided the most active compounds in series 4, with better potency than their congeners in series 2 and equal potency to those in series 1. The few compounds available for direct comparisons between series 4 and series 1 indicate that the potency of compounds with aromatics at R_1 and cycloalkyls at R_2 in general favored series 1. Large bis-cycloalkyl-substituted compounds are of similar potency in congeners of both series (**4rr**, **4tt**, **4uu**), while the smaller cyclopentyl (**4ss**) is substantially less potent in series 4. The available comparisons with series 2 compounds reveal that the potency of compounds with cycloalkyls at R_1 and aromatics at R_2 (**4e**, **4g**, **4i**, **4k**, **4m**, **4y**) were equivalent in the two series, while the reverse combinations or bis- or mixed cycloalkyl substitutions favored series 4 (**4z**, **4rr**, **4uu**, **4ww**, **4zz**). Series 3 compounds were less potent

than congeners in series 4 when comparing cycloalkyls/aromatics at R_1/R_2 (**4e**, **4k**), whereas similar activity resulted when comparing bis-phenyl or phenyl/cycloalkyl congeners (**4a**, **4o**). Substitution with smaller cycloalkyls favored series 4 compounds (**4rr**, **4ss**) but larger alkyls were comparable in series 3 and 4 (**4tt**, **4uu**). Combinations of cycloalkyls again favored series 4 over series 3 when smaller groups or bicycloheptane were inserted in R_2 (**4ww**, **4vv**), but again the distinction is lost when more bulky groups are inserted in R_2 (**4zz**).

Because of their high potency when attached to the amide linkages that characterize series 4, cycloalkyl combinations were studied in detail. As noted with other series, a comparison of the activity of mixed aromatics and cycloalkyls favored larger alkyl groups such as adamantyl over cyclohexyl or bicycloheptyl (**4e**, **4k**, **4n**). Phenyls substituted with electron withdrawing nitro moieties (**4h**) were of equivalent potency to the unsubstituted aryls (**4e**, **4k**), which were of lower potency that halogen-substituted phenyls (**4g**, **4l**, **4m**). A mixture of R_1 aryl and R_2 alkyl (**4o**) is less active than the reverse (**4k**). Azide substitution on the 4-position of the phenyl in R_1 (**4p**) is considerably more potent than amino substitutions (**4q**). When 2-amino pyridines were used in combination with bulky aliphatic group in either orientation, the resulting compounds suppressed IgE with moderate-high potency (**4z**, **4y**). However, as noted in series 2, potency was reduced when 3- or 4-aminopyridines were used at R_1 or R_2 (**4aa**, **4bb**) in place of 2-pyridine (**4z**). Dimethyl substitution on the 2-pyridine improved the potency over unsubstituted 2-pyridine (**4mm**). When paired with 2-pyridine at R_1, adamantyl at R_2 is more potent than smaller alkyls (**4cc**, **4ff**). Thus, substitutions were made on the adamantyl at R_2 in an effort to improve solubility without sacrificing potency. Compounds with acetylated or 2-OH, or 3-OH adamantane (**4gg**, **4hh**, **4nn**) were of lower potency, while 4-OH- or halo-substituted adamantyls (**4ii**, **4nn**, **4kk**, **4ll**) were more potent than unsubstituted adamantane (**4z**). Combination of 2-pyridine at R_1 with the 4-O-methyloxime of adamantane at R_2 yielded a compound (**4jj**) with moderate potency, but toxicity was a concern. Insertion of hydrogen or methyl in R_1 or R_2 resulted in a substantial loss of activity (**4oo**, **4pp**, **4qq**). As with other series, bis-cycloalkyls of series 4 display a preference for bulkier terminal structures (**4rr**, **4ss**, **4tt**, **4uu**). Mixtures of cycloalkyls (adamantyl, cyclohexyl, or bicycloheptyl) appeared to demonstrate little preference for R_1 versus R_2 in series 4 (**4vv**, **4ww**, **4xx**, **4yy**, **4zz**, **4aaa**, **4bbb**).

A further effort to enhance potency and/or hydrophilicity focused on substitutions in the phenyl ring Y. Several compounds having the combination of 2-pyridine at R_1 and adamantyl at R_2, and with substitutions on the Y phenyl allow a direct comparison of the impact of the latter substitutions. 2-Fluoro (**4fff**) or 3-fluoro (**4ggg**) substitution on the aromatic ring (Y) retained or reduced potency, respectively, compared with its unsubstituted parent (**4z**). Potency was substantially reduced by substituting 2-CF_3 (**4hhh**). Hydroxyl substitution either at the 2-Y (**4eee**) or 3-Y (**4iii**) position did not affect the relative potency of their counterparts that lack the Y-substitution (**4z**). In comparison to the unsubstituted adamantyl at R_2 (**4iii**), 3-OH-adamantyl (**4jjj**) and 3-acetoxy adamantyl (**4kkk**) were more potent. When 2-F was substituted in the Y position little change in potency was observed for either the 4-keto- or 4-hydroxyl-adamantyl compounds (**4iii**, **4mmm**). However, a greater than 10-fold loss of potency resulted from substituting a hydroxyl in the 3-position of the adamantyl (**4nnn**).

In summary, although there are series-specific variations, compounds of all series generally share the same SARs. Of the four series of compounds, series 1 are the most potent followed by series 4, series 2, and then series 3. This is revealed by direct comparisons of the IgE IC50s of congeners that are present in each pair of series: i.e. series 1 versus series 2, 12 vs. 58 nM (number of available direct comparisons [n] = 8); series 1 versus series 3, 18 vs. 388 nM (n = 8); series 1 versus series 4, 8 vs. 40 nM (n = 10); series 2 versus series 3, 30 vs. 58 nM (n = 7); series 2 versus series 4, 22 vs. 7 nM (n = 11); and series 3 versus series 4, 92 vs. 32 nM (n = 10). Cross-series comparisons also reveal that compounds with small aliphatic or aromatic groups at R_1 or R_2 exhibit low potency (IC_{50} > 90 nM) (**2d**, **3j**, **3k**, **3l**, **4pp**, **4qq**), but this significantly improves when aliphatic cyclic-alkyl groups or di-halogenated aromatic rings are incorporated at R_1 and R_2 in bis (e.g. **1h**) or in combinations (**1gg**, **2j**, **3g**, **4g**). Symmetrical and non-symmetrical bis-acyl or aryl amides often resulted in potent compounds (**1h**, **1mm**, **1nn**, **2cc**, **3f**, **4dd**), but their oral bioavailability (as described below) was low. Electron donating aromatic groups or hetero-aryl groups at R_1 and R_2 resulted in low activity (**1z**, **2c**, **2o**, **4c**). When the imidazole hydrogen was blocked with small aliphatic chains or even CH_3, the potency decreased significantly (IC_{50} = 2000 nM) (**1ww**, **1xx**) indicating that imidazole free hydrogen is crucial for activity. The impact of the systemic bioavailability after oral administration of these compounds is integrated into the discussion below.

3.4. Selecting the lead compound

Small molecule drugs for the treatment of chronic diseases optimally should be active following oral administration, and thus systemic bioavailability after oral administration must be factored into the SAR analysis. Moreover, although in vitro systems exist to estimate this character, the intact animal allows for a more accurate assessment of oral bioavailability since serum levels of drug also reflect the impact of the "first pass" effect as the absorbed compound passes through the liver into the bloodstream. Although series 1 compounds achieved the highest potency as a group, these highly potent halogen-substituted aryls (**1ee**, **1gg**) and large bis cyclo-alkyls (**1mm**, **1nn**, **1oo**) were poorly bioavailable following oral administration. On the other hand, series 4 emerged as the group with the highest serum bioactivity (determined by dividing the peak serum concentration by the IC50) after oral administration. Thus bioavailability data are shown for series 4 (Table 4), while the analyses of series 1–3 are limited to selected comparisons in Table 5 and a discussion in the text.

Pharmacological activity of the 2-phenyl benzimidazole compounds is dependent on the lipophilicity of the terminal substitutions, a character that negatively affects aqueous solubility and oral absorption. This is evident in the bioavailability data described above and provided in Table 4. The most potent compounds such as those containing bis-cycloalkyls or bis-aryls achieved very low serum levels, while compounds substituted with hetero-aryls (**4c**) or containing polar groups such as O-methyloxime (**4jj**) achieved high serum concentrations but exhibited low potency. To improve serum bioactivity following oral administration a strategy was needed that would incorporate features of the molecule that translate to potency on one hand while improving solubility on the other. Because large cycloalkyl groups appear to contribute the most to potency, they were paired in various combinations with bulky but more hydrophilic groups.

Acyl and aryl combinations at R_1 and R_2 provided the best series 4 compounds, with better potency than their congeners in series 2 and with equivalent potency and better oral bioavailability than those in series 1. To improve dissolution and oral bioavailability, several combinations of adamantyl groups and hetero-aryls were tested. When 2-amino pyridines were used at R_1 in combination with a bulky aliphatic group at R_2 (**4z**), a compound with moderate potency and acceptable oral bioavailability resulted (Table 4). Reversing the groups retained the potency but drastically reduced oral bioavailability (**4y**). Attempts to improve hydrophilicity by substituting the terminal adamantyl with hydroxyl or keto groups (**4gg**, **4ii**, **4iii**, **4jjj**, **4kkk**, **4lll**) resulted in a loss of oral bioavailability likely because they were readily conjugated. The fluoro substituted compounds (**4fff**, **4ggg**) provided similar or reduced in vitro potency, and provided a modestly improved serum concentration profile over their unsubstituted parent (**4z**). The best oral bioavailability was obtained by coupling 2-OCF_3-substituted phenyl at R_2 with the 2-pyridine at R_1 (**4c**). Thus, although incorporation of hetero-aryl groups (S or N) at R_1 and R_2 resulted in less active compounds, they improved the dissolution rate and solubility, thereby increasing oral bioavailability. Moreover, they are amenable to preparing salts (**4z**).

When factoring the in vitro potency of these compounds, **4z** and **4fff** achieve the highest serum bioactivity after oral administration. This is shown in Table 5, which compares the influence of halogen substitution, the substitution site of the pyridine, and the orientation of the amide linkages. Comparisons I–IV show that o-halo-substituted phenyls promote improved bioavailability over *p*-substitution in series 4 but not in series 1 compounds (**1ii**, **1jj**, **4r**, **4v**, **4s**, **4t**, **4u**, **4w**, **4x**), although the loss of bioavailability by the *p*-substitution is at least partially compensated by an increase of potency (**4u**, **4x**). Comparison II shows that o-OCF_3 substitutions provide better oral bioavailability than o-F in series 4 compounds (**4s**, **4t**). Comparison IV shows the benefit of the 2-pyridines over the 3- and 4-pyridines (**4z**, **4aa**, **4bb**), while comparison V demonstrates the superiority of series 4 over the other series when comparing serum bioactivity of compounds containing 2-pyridine at R_1 and 1-adamantyl at R_2 (**1kk**, **2v**, **3aa**, **4z**). Finally, substitution of fluorine on the Y phenyl (comparison VI) improves oral bioavailability but caused little change in potency (**4fff**, **4ggg**). However, the fluorinated compounds showed a tendency for increased toxicity in vivo and thus were not considered for further evaluation.

On the strength of its bioactivity following oral dosing and lower toxicity, **4z** was selected as the lead compound. The oral bioavailability of **4z** was further augmented by making its bis-methansulfonic salt, resulting in a threefold increase of the peak serum concentration after oral dosing. The 4-hydroxylated adamantane compound (**4ii**) was found to be a prominent active metabolite of this compound.

3.5. IgE response in human peripheral blood lymphocytes

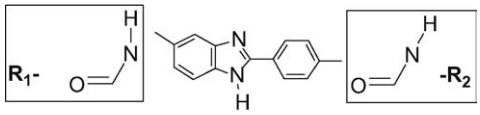
To examine the breadth of their pharmacological profile, compounds from each structural series were selected for additional testing and comparison with mouse IgE response. To demonstrate that the effect on IgE translates across species barriers, lymphocytes were isolated from fresh human peripheral blood (PBL) and cultured with human IL-4 and anti-human CD40 antibody for 12 days. Thus the assay was carried out similar to the in vitro IgE assay in mouse cells. Suppression of IgE production was observed in parallel cultures that contained 2-phenyl benzimidazole derivatives (Table 6) and with similar potencies as observed in the in vitro mouse model (r^2 linear goodness-of-fit = >0.99). These results suggest that the target of these compounds performs a similar role in both mouse and human lymphocytes.

3.6. In vivo antibody response

To assess whether the in vitro results translate to the intact animal, BALB/c mice were subjected to an in vivo antigen challenge model while receiving selected compounds from the 2-phenyl benzimidazole series. As a secondary screening tool, it was necessary that this procedure be short, not labor-intensive, and require a minimum quantity of compound. Thus, a protocol was chosen that employed T cell priming in irradiated mice followed by challenge with specific antigen during a 3-day course of drug [57,58]. Three weeks following antigen challenge, mice were bled and serum antibody concentrations determined. Irradiation is known to preferentially enhance the IgE response while the combination of T cell priming and antigen challenge provides a robust IgE stimulus, an important issue when generating an IgE response in an animal that is naturally resistant to developing allergies. Moreover, because the mice were not exposed to antigen prior to drug treatment this minimizes the background problem that would occur if mice were primed with specific antigen instead of the carrier protein alone. This further expedites compound testing during the discovery phase of development.

Intraperitoneal (i.p.) administration of **1mm** suppressed the IgE response to specific antigen (DNP) while not affecting DNP-specific IgG1 and IgG2a levels in the serum of primed BALB/c mice 20 days following immunization with DNP_6-

Table 6
Comparison of potencies versus mouse IgE, human IgE, cytokines, and CD23



A B

Series	A	B
1	-CO-NH-	-NH-CO-
2	-NH-CO-	-CO-NH
3	-CO-NH-	-CO-NH-
4	-NH-CO-	-NH-CO-

Compounds	R1	R2	Mouse IgE[a] IC50 (nM)	Human IgE[b] IC50 (nM)	IL-4[c] IC50 (nM)	IL-5[c] IC50 (nM)	CD23[d] IC50 (nM)
1mm	Cyclohexyl	Cyclohexyl	5	2	2.5	6	25
1nn	1-Adamantyl	1-Adamantyl	4	3	2	12	30
1gg	Cyclohexyl	3,4-Dichlorophenyl	0.8	0.8	0.4	4	15
2x	Cyclohexyl	Cyclohexyl	80	50	30	60	525
2y	1-Adamantyl	1-Adamantyl	16	25	10	45	250
2v	2-Pyridine	1-Adamantyl	40	40	15	40	400
3n	1-Adamantyl	1-Adamantyl	6	8	3	12	50
3m	Cyclohexyl	Cyclohexyl	50	35	12	60	400
3a	Phenyl	Phenyl	400	250	180	225	> 2000
4rr	Cyclohexyl	Cyclohexyl	9	8	5	12	80
4z	2-Pyridine	1-Adamantyl	8	10	4	20	80
4uu	1-Adamantyl	1-Adamantyl	5	4	2	15	35
4i	1-Adamantyl	3,4-Dichlorophenyl	2	1.5	0.4	5	7

[a] In vitro IgE from Tables 1 to 4.
[b] Human IgE response measured in PBL.
[c] ConA-stimulated murine IL-4 and IL-5.
[d] Murine spleen cell CD23.

KLH/alum (Fig. 1A). Dexamethasone also depressed the IgE response to specific antigen similar to previous reports but exhibits no selectivity over its effect on DNP-specific IgG1 or IgG2a. Although not shown, similar results were achieved with **4z** as observed for **1mm**, and follow-up experiments have shown the ED50 for **1mm** and **4z** to be 1 and 2 mg/kg per day i.p., respectively. The potency of 53 phenyl benzimidazole derivatives tested in both the ex vivo (IC50) and in vivo (ED50) IgE response assays were compared in Fig. 1B. Although many of the in vivo ED50 values are estimates based on partial responses, the result demonstrates a positive relationship between IgE responses in vitro and in vivo. This validates the ex vivo screening assay as a means for identifying compounds with the capacity to suppress antigen-specific IgE response in the intact animal.

ED_{50}s generated by this approach, however, are somewhat artificial in that our protocol provides drug over a 3–4-day period that is completed 2–3 weeks prior to measuring serum IgE. This delay between dosing and IgE measurement provides an intriguing insight into its possible mechanism. Because the compounds are eliminated within 24 h of dosing and minimal IgE is found in the serum of control animals during the first week following antigen challenge (not shown), this suggests that the benzimidazole compounds probably do not act on IgE synthesis/release in this model. This implicates drug action on an earlier stage such as antigen presentation, or a T cell function such as cytokine production.

Finally, it should be pointed out that 2-phenyl benzimidazole compounds are capable of suppressing the level of IgE in this in vivo assay to only 20% of that achieved in vehicle-treated mice. While this might be expected to provide some benefit for controlling allergic manifestations in the clinical environment, it has nonetheless been demonstrated that greater than 95% reduction of circulating IgE is required for significant therapeutic benefit from the anti-IgE antibody omalizumab [15,16]. Moreover, we have further shown that **1mm** is effective in suppressing asthma in an animal model in doses that do not quantitatively affect antigen-specific IgE [55]. This issue was directly addressed by an additional experiment that tested the response to exogenous administration of IgE–antigen complexes (anti-DNP IgE – DNP-OVA) to OVA-sensitized mice. Sensitized mice experienced strong pulmonary eosinophil infiltration in response to intranasal instillation of IgE–antigen complexes, and this was inhibited by administration of **1mm** (data not shown). These results suggest that 2-substituted benzimidazole compounds suppress airway responses to allergen challenge beyond their effect on IgE production/release. Thus while these compounds clearly suppress IgE responses in specific protocols, the modest reduction of IgE in mice treated with 2-phenyl benzimidazole derivatives suggests that the

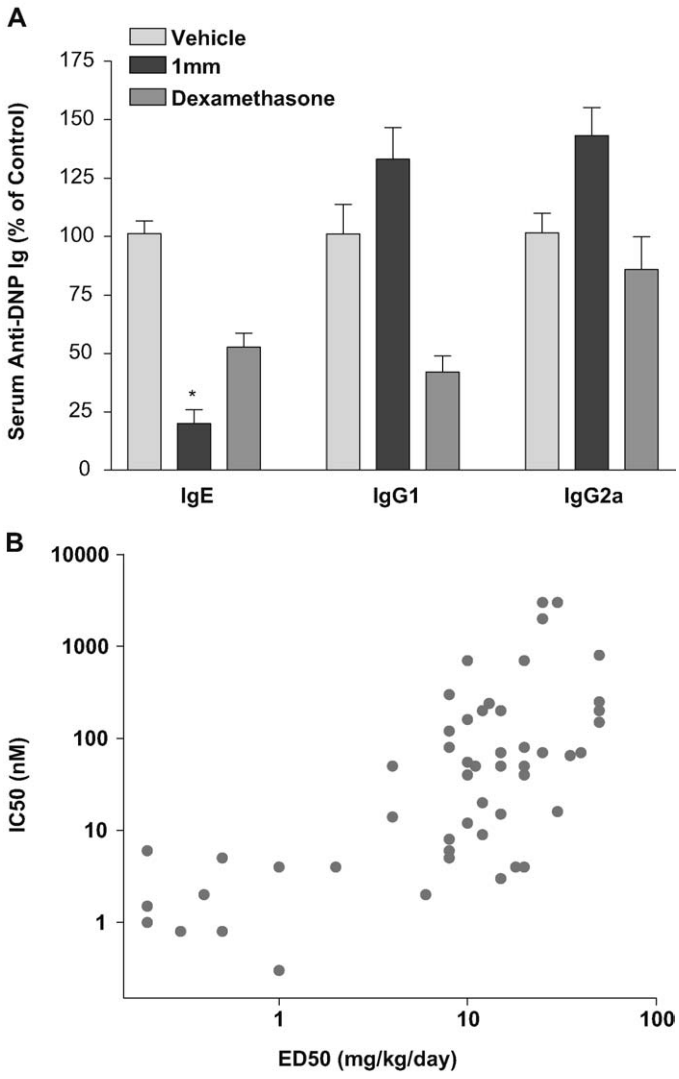


Fig. 1. Antigen-specific antibody responses in vivo. **A**. Female BALB/c mice (5–10 mice per group) were irradiated (250R), primed with KLH/alum, and 7 days later challenged with DNP_6-KLH/alum. Four doses of either **1mm** (20 mg/kg), dexamethasone (100 μg per injection), or vehicle (0.25% methylcellulose) were administered i.p. over a 3-day period starting 1 day prior to antigen challenge. Three weeks following challenge with DNP_6-KLH, the mice were bled and DNP-specific IgE, IgG1, and IgG2a were quantified by ELISA. Control mice produced serum levels of approximately 15, 1500, and 3 μg/ml of DNP-specific IgE, IgG1, and IgG2a, respectively. Subsequent dose–response studies have yielded an ED50 for **1mm** of 1 mg/kg per day for suppression of IgE; * $P < 0.01$ by Bonferroni's multiple comparison test. **B**. The potencies of 53 2-phenyl benzimidazole compounds in the ex vivo (IC_{50}, nM) and in vivo (ED50, mg/kg per day i.p.) IgE response assays are compared.

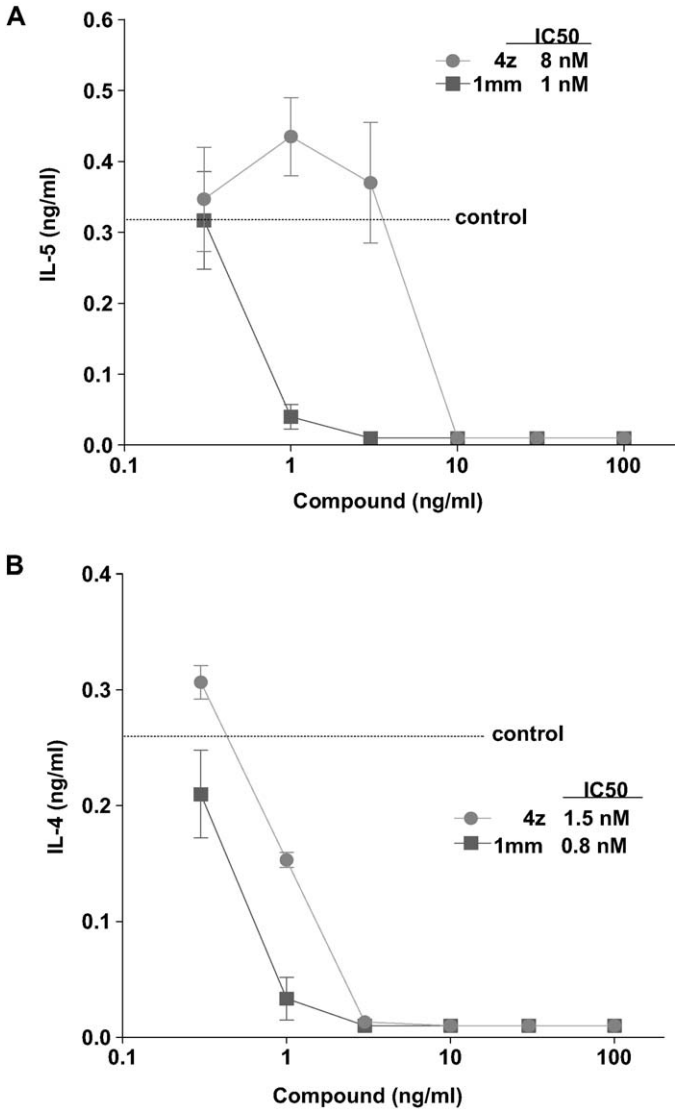


Fig. 2. IL-4 and IL-5 responses in spleen cells from antigen-sensitized mice. Spleens were removed from female BALB/c mice that had been sensitized by i. p. injection of DNP_6-KLH. Cell cultures were established in the presence of DNP_6-KLH and drug, as noted for the ex vivo IgE response assay. After 4 days of culture, ConA (5 μg/ml) was added and the cells cultured for an additional 4 h. IL-4 and IL-5 levels in the culture supernatants were quantified by ELISA (Panel **A** and **B**, respectively). Control responses were determined from cultures that received stimulus but contained no drug.

anti-atopic activity of these agents are not dependent on IgE suppression.

3.7. Cytokine response

In the in vitro IgE response assay, the two stimuli minimally required by B cells to produce IgE are provided exogenously, including cytokine (either IL-4 or IL-13) and CD40 engagement (anti-CD40 antibody). Thus the 2-phenyl benzimidazole compounds act directly on B cells to block IgE production/release. However, the higher potency displayed by most of these compounds in the ex vivo relative to the in vitro assay suggests that in addition to the B cell, they are acting on accessory cells as well. Since T cells comprise about half the population of lymphocytes in BALB/c mouse spleen, these cells are likely the source of both the cytokines and CD40 ligand (CD154). A similar response is observed when substituting bacterial lipopolysaccharide (LPS), a co-stimulatory molecule commonly used for initiating polyclonal IgE responses in vitro [59,60], for the CD40 engagement in the in vitro IgE assay (data not shown). Because the inhibitory potencies of 2-phenyl benzimidazoles are similar regardless of whether anti-CD40 antibody

or LPS are used, this suggests that these compounds may prevent cytokine release by the T cell.

To directly examine the activity of 2-phenyl benzimidazole compounds against cytokine response in T cells, two assays were developed with BALB/c mouse spleen cells. Fig. 2 shows the inhibitory activity of **1mm** and **4z** against the responses of IL-4 and IL-5 to the combination of specific antigen (DNP_6-KLH) and ConA. This assay closely follows the ex vivo IgE response assay except that ConA is added during the final 48 h to boost the cytokine response. Cultures containing antigen alone or ConA alone (during the last 48 h) yielded low and variable cytokine levels, as measurable by ELISA.

A second assay was established with isolated T cells from the spleens of naive mice using ConA alone as stimulus. The culture period was shortened to 40 h to minimize secondary cytokine responses to other mediators that might have been released into the culture. To enhance the sensitivity of the assay, IL-4 and IL-5 were detected using fluorescent beads and quantified by a Luminex 100 flow analyzer. Examination of cytokine responses in isolated T cells thus provides a less ambiguous means for evaluating direct effects on T cell. As performed above for human IgE responses, selected compounds from each of the four structural series were tested for activity against IL-4 and IL-5 responses (Table 6). All compounds suppressed both cytokines with potencies that paralleled the results with the ex vivo cytokine assay and the in vitro IgE responses in mouse cells. Plotting the IC50s for inhibition of mouse IgE and IL-4 (or IgE and IL-5) for the compounds in Table 6 yielded a r^2 linear goodness-of-fit value of > 0.99. Inhibition of cytokine responses by T cells isolated from mouse spleen indicates that in addition to a B cell-specific action, 2-phenyl benzimidazole compounds also act directly on the T cell. Moreover, the similar potency that the 2-(substituted phenyl)-benzimidazole derivatives exhibit against B cell IgE and T cell cytokine responses suggest that all compounds are acting on the same target for both responses and in both cell types. Interestingly, as observed for the IgE response assays, the potency against IL-4 and IL-5 in the in vitro assay with naive cells is about threefold lower than for the ex vivo assay with sensitized cells. Thus it is possible that in an assay system that requires more than a single cell type, the effects of 2-phenyl benzimidazole compounds on each cell type involved in the measured response are additive.

3.8. CD23 expression

IgE and cytokines are secreted proteins, and their inhibition by the 2-(substituted phenyl)-benzimidazole derivatives irrespective of the stimuli suggests that the compounds are acting downstream of the inductive pathway. To assess whether these compounds perturb the expression of molecules at the plasma membrane, their effect on the expression of CD23 was tested. CD23 is the low affinity receptor for IgE expressed on dendritic cells, macrophages, B cells, and eosinophils [61], and involved in the regulation of IgE, presentation of antigen, and airway responsiveness [18–26]. Moreover, CD23 is regulated

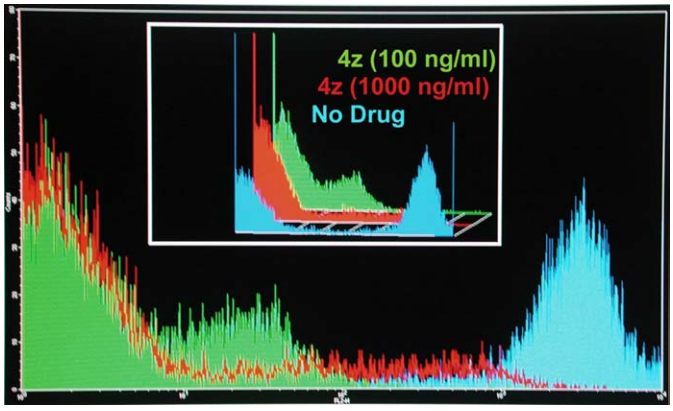


Fig. 3. CD23 expression on mouse spleen cells. BALB/c mouse spleen cells were depleted of red blood cells and cultured in the presence of IL-4 (10 ng/ml) and anti-CD40 Ab (100 ng/ml), with or without **4z** (100 or 1000 ng/ml). After 24 h, cells were harvested, washed, stained with anti-CD23 antibody, and analyzed by flow cytometry. The histograms of the control and drug-treated samples are overlaid: control (blue shaded), **4z** 100 ng/ml (green shaded), **4z** 1000 ng/ml (red line). The inset shows the original histograms.

in parallel with IgE, requiring CD40 engagement and either IL-4 or IL-13 for maximal expression [62,63]. To test for effects on CD23 expression, spleen cells from BALB/c mice were depleted of red blood cells and cultured with IL-4 and anti-CD40 antibody for 24 h. Cells cultured in the presence of **4z** showed a concentration-dependent suppression of CD23 on their cell surface (Fig. 3). Repeating this assay in the presence of a full concentration range of all compounds (Table 6) resulted in CD23 suppression with IC_{50}s that paralleled those required for inhibition of mouse IgE (r^2 linear goodness-of-fit, > 0.98).

The similar potency of all compounds against multiple responses in different cells (Table 6) suggests that a highly conserved target is integral to the response in all assay systems. Moreover, the target for these compounds does not reside in a compartment dedicated to protein secretion but perhaps in an upstream compartment such as the Golgi or endoplasmic reticulum. Indeed, separate work has identified the Golgi as the likely site of action for the 2-(substituted phenyl)-benzimidazole derivatives (data not shown). These compounds, moreover, act via a mechanism that is clearly distinct from other agents (such as brefeldin A and monensin) known to disrupt this organelle. This provides a conceptual basis in which to frame the diverse responses to these compounds.

4. Conclusions

A comparison of four novel series of 2-(substituted phenyl)-benzimidazole derivatives reveal a distinct SAR for the inhibition of IgE response in vitro. In addition to the core 2-(substituted phenyl)-benzimidazole backbone, the active structure has a strong preference for terminal large lipophilic groups such as aliphatic cycloalkyls or aromatics flanking the amide linkages. Compounds from series 1 were favored for potency but series 4 compounds showed better serum bioactivity after oral dosing, and a compound from the latter group (**4z**, AVP-13358) was

chosen as a development candidate for evaluation in clinical trials. The molecular target of these compounds has not been characterized, although they act directly on B cells to block secretion of IgE and expression of CD23, and directly on T cells to inhibit the release of IL-4 and IL-5. Moreover, these diverse responses were suppressed with similar potency suggesting that a common target mediates all pharmacological activities. Their broad-based activity against several allergy markers and their novel mechanism of action thus suggests that these compounds may offer important new tools for the treatment of allergic disorders.

5. Experimental protocols

The analogs were made according to following process for each series. General preparation of lead compounds from each series- 1–4 are discussed as follows.

5.1. Syntheses

5.1.1. Method A (series 1): cyclohexanecarboxylic acid [4-(5-cyclohexylamino-1H-benzoimidazol-2-yl)-phenyl]-amide (**1mm**, Y = H)

5.1.1.1. 4-(5-Nitro-1H-benzoimidazol-2-yl)-phenylamine (7, Y = H). To a suspension of polyphosphoric acid (115 g) in xylene (100 ml) at 60 °C, 4-nitro-1,2 phenylenediamine (**5**) (11.2 g, 73.2 mmol) and 10.2 g (74.3 mmol) 4-aminobenzoic acid (**6**) were added. The temperature was raised to 130 °C for 1 h before it was raised to 150 °C and stirred for 6 h. The reaction mixture was cooled and diluted with hot water with stirring. The hot reaction mixture was filtered through a Buchner funnel and solid was isolated. Mother liquor also afforded additional solid. Both solids were taken in water (500 ml) and neutralized with solid $NaHCO_3$. The yellowish green solid was filtered and washed with hot water (5 × 500 ml). The solid was crystallized from hot THF (600 ml). This process yielded a yellow solid (**7**) (8.7 g, 47%), m.p. 283–285 °C; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 13.14 (br s, 1H), 8.38 (br s, 1H), 8.16 (d, J = 3.2 Hz, 1H), 7.98 (d, J = 2.4 Hz, 2H) 7.68 (br s, 1H), 6.88 (d, J = 5.6 Hz, 2H), 5.87 (br s, 2H); ESI-MS (m/z): 255.6 (M + H).

*5.1.1.2. Cyclohexanecarboxylic acid [4-(5-nitro-1H-benzoimidazol-2-yl)-phenyl]-amide (**8**, R_2 = cyclohexyl, Y = H).* To a solution of **7** (1.26 g, 4.97 mmol) in anhydrous pyridine (10 ml) at room temperature, cylohexane carbonyl chloride (1.0 ml, 5.14 mmol) was added and the reaction mixture was stirred at room temperature for 3 h, quenched with water, and solid was filtered. The gummy solid was taken in EtOH (0.020 ml) and stirred with 10% NaOH (1 ml) at 90 °C for 30 min. The EtOH was evaporated and water was added. The solid **8** was filtered, washed with water, and dried (1.36 g, 73%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 13.60 (br s, 1H), 10.18 (br s, 1H), 8.48 (br s, 1H), 8.14 (br d, J = 12 Hz, 2H), 8.10 (d, J = 8.0 Hz, 1H), 7.82 (d, J = 8.0 Hz, 2H), 7.73 (br s, 1H), 2.45 (m, 1H), 1.87–1.65 (m, 5H), 1.48–1.20 (m, 5H); ESI-MS (m/z): 365 (M + H).

*5.1.1.3. Cyclohexanecarboxylic acid [4-(5-amino-1H-benzoimidazol-2-yl)-phenyl]-amide (**9**, R_2 = cyclohexyl, Y = H).* A solution of **8** (1.25 g, 3.43 mmol) in MeOH/THF (8:2; 40 ml) was added with 5% Pd/C and the reaction mixture was stirred under H_2 at 80 °C for 16 h. The amine **9** was freed from catalyst and dried under vacuum (1.09 g; 95%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.11 (br s, 1H), 10.07 (s, 1H), 8.06 (d, J = 8.80 Hz, 2H), 7.72 (d, J = 8.80 Hz, 2H), 7.29 (br s, 1H), 4.95 (br s, 2H), 2.39–2.31 (m, 1H), 1.88–1.60 (m, 5H), 1.50–2.15 (m, 5H); ESI-MS (m/z): 335.6 (M + H).

*5.1.1.4. Cyclohexanecarboxylic acid [4-(5-cyclohexylamino-1H-benzoimidazol-2-yl)-phenyl]-amide (**1mm**, Y = H).* Cyclohexane carbonyl chloride (0.650 g, 4.5 mmol) was added to a stirring solution of amine **9** (1.00 g, 3 mmol) in anhydrous THF (15 ml) and pyridine (5 ml), and the reaction mixture stirred at room temperature for 16 h. The reaction mixture was poured into water and the solid (**1mm**) was filtered and re-crystallized from EtOAc/hexane, m.p. 336–337 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 12.6 (br s, 1H), 10.1 (s, 1H), 9.8 (s, 1H), 8.15 (br s, 3H), 7.80 (br s, 2H), 7.45 (br s, 1H), 7.25 (br s, 1H), 2.34 (m, 2H), 1.90–1.10 (m, 22H). Analysis: $C_{27}H_{32}N_4O_2 \cdot 0.60\ H_2O$ (C = 71.13; H = 7.19 N = 12.14).

5.1.2. Method B (series 2): 2-(4-cyclohexylcarbamoyl-phenyl)-1H-benzoimidazole-5-carboxylic acid cyclohexylamide (**2f**, Y = H)

*5.1.2.1. 2-(4-Methoxycarbonyl)-phenyl)-1H-benzimidazole-5-carboxylic acid (**12**, Y = H).* A mixture of 3,4-diaminobenzoic acid (**10**) (3.0 g, (19.7 mmol) and methyl-4-formyl benzoate (**11**) (3.24 g, 19.7 mmol) in nitrobenzene (80 ml) was heated at 150–155 °C for 18 h. The reaction mixture was cooled to room temperature and diluted with Et_2O (500 ml). The solids were filtered, washed with Et_2O, and vacuum dried to get 3.2 g solid **12** (3.2 g, 52% yield); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 13.40 (br s, 1H), 8.33 (br d, J = 7.60 Hz, 2H), 8.21 (br s, 1H), 8.13 (br d, J = 7.60 Hz, 2H), 7.94 (br s, 1H), 7.87–7.80 (dd, J = 20.0 and 8.0 Hz, 1H), 7.69 (br d, J = 20.0 Hz, 1H), 3.91 (s, 3H); ESI-MS (m/z): 297.4 (M + H).

5.1.2.2. 4-(5-Cyclohexylcarbamoyl-1H-benzoimidazol-2-yl)-benzoic acid methyl ester (13, R_1 = cyclohexyl, Y = H). A solution of acid **12** (5.0 g, 16.9 mmol) in dry DMF (250 ml) and CDI (7.1 g, 44 mmol, 2.5 eq.), was stirred at room temperature for 3 h. Cyclohexyl amine (2.0 g, 23 mmol, 1.35 eq.) was added, refluxed for 96 h, poured into water (2.0 l), and stirred at room temperature for 16 h. The resulting solid was filtered and washed with water. The solid **13** was re-crystallized from THF–MeOH–Et_2O (0.4 g, 6% yield) and used for the next step.

*5.1.2.3. 4-(5-Cyclohexylcarbamoy)-1H-benz imidazol-2yl)benzoic acid (**14**, R_1 = cyclohexyl, Y = H)*. A mixture of methyl 4-(5-(cyclohexylcarbonyl)-1H-benzoimidazole-2-yl)benzoate (**13**) (2.5 g, 6.6 mmol) in THF (30 ml), water (25 ml), and LiOH (1.06 g) was stirred at room temperature for 2 h, mixed with charcoal, stirred with slight heating, and then filtered. The filtrate was chilled in ice and acidified with concentrated HCl to pH 1.0. The acid **14** was filtered, washed with water and re-crystallized from THF/MeOH/Et_2O to get a white solid (1.9 g, 80%); m.p. 275–280 °C and used without further purification for the next step.

*5.1.2.4. 2-(4-Cyclohexylcarbamoyl-phenyl)-1H-benzoimidazole-5-carboxylic acid cyclohexylamide (**2f**, Y = H)*. A reaction mixture of acid **14** (0.2 g, 0.56 mmol) in anhydrous DMF (2.0 ml), EDCI (0.107 g, 1 mmol), HOBt (0.076 g, 1 mmol), and Et_3N (0.3 ml) was stirred at room temperature for 5 min. A solution of aniline (0.052 g, 1 mmol) in DMF (0.015 ml) was added and stirred for 21 h. The reaction was quenched with ice water (0.010 ml) and the solids were filtered and washed with ice water. The solids were re-crystallized from EtOH/H_2O as being solid of **2f** (0.168 g, 34%); m.p. 324–325 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 13.27 (br s, 1H), 10.37(s, 1H), 8.46–8.13 (br m, 6H), 7.90–7.70 (br m, 4H), 7.41–7.34 (t, J = 9.0 Hz, 2H), 7.16–7.09 (m, 1H), 3.80 (m, 1H), 1.84–1.78 (br m, 5H), 1.34–1.05 (br m, 5H). Analysis: $C_{27}H_{26}N_4O_2 \cdot 1.68\ H_2O$ (C = 69.17; H = 6.14; N = 12.01).

*5.1.3. Method C (series 3): 4-[5-(cyclohexanecarbonyl-amino)-1H-benzoimidazol-2-yl]-N-cyclohexyl-benzamide (**3m**, Y = H)*

*5.1.3.1. 4-(5-Nitro-1H-benzoimidazol-2-yl)-benzoic acid methyl ester (**15**, Y = CH_3)*. A mixture of 4 nitrophenylene-1,2-diamine (**5**) (6.34 g, 41.4 mmol) and methyl 4-formyl-benzoate (**11**) (6.80 g, 41.4 mmol) was heated in nitrobenzene (100 ml) at 155–160 °C for 18 h. The reaction mixture was cooled to room temperature and diluted with Et_2O (500 ml), and solid **15** was filtered and washed with ether (8.0 g, 56%).

*5.1.3.2. N-Cyclohexyl-4-(5-nitro-1H-benzoimidazol-2-yl)-benzamide (**17**, R_2 = cyclohexyl, Y = H)*. To a solution of nitro ester **15** (8.0 g, 26.9 mmol) in THF (27 ml) and water (26 ml), LiOH (3.39 g) was added and stirred at room temperature until the hydrolysis was complete. The solvent was evaporated and ice water was added. The reaction mixture was acidified with 3 N HCl and solid was filtered, washed with water, dissolved in hot DMF and chilled to 4 °C. The yield of the acid **16** was 5.37 g (70%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 13.80 (br s, 1H), 13.20 (br s, 1H), 8.55 (br s, 1H), 8.34 (m, 2H), 8.15 (m, 2H), 7.95 (br d, J = 16.0 Hz, 1H), 7.81 (br s, 1H); ESI-MS (m/z): 284.3 (M + H).

To a solution of acid **16** (2.0 g, 7.0 mmol) in DMF (8.0 ml), CDI (1.70 g, 10.5 mmol) was added and stirred at room temperature for 2 h. Cyclohexyl amine (1.60 ml, 14 mmol) was added to the reaction mixture and heated at 100 °C for 18 h. The reaction mixture was poured into ice water (100 ml), and stirred at room temperature for 4–5 h. The nitro-amide **17** was filtered, washed with water and ether and dried in vacuum (yield 1.9 g, 71%) and used without further purification for the next step.

*5.1.3.3. 4-(5-Amino-1H-benzoimidazol-2-yl)-N-cyclohexyl-benzamide (**18**, R_2 = cyclohexyl, Y = H)*. The nitrocyclohexylamide **17** (1.9 g, 5.0 mmol) was hydrogenated in presence of 5% Pd–C (0.475 g) in MeOH (40 ml) at room temperature for 18 h. The amine **18** was filtered, and solvent was evaporated. The residue was treated with ether (20 ml), and solid was filtered, re-crystallized from THF/MeOH/hexane and dried in vacuum. Yield: 1.05 g, 60%.

*5.1.3.4. 4-[5-(Cyclohexanecarbonyl-amino)-1H-benzoimidazol-2-yl]-N-cyclohexyl-benzamide (**3m**, Y = H)*. A mixture of amine **18** (0.5 g, 1.50 mmol) in dry THF and pyridine (0.54 g) was heated to a clear solution. Then a solution of cyclohexyl carbonyl chloride (0.26 g, 1.77 mmol) in THF (10 ml) was added and refluxed for 18 h. The reaction was poured on water and stirred for 1 h. The solid **3m** was filtered, washed with water and ether, re-crystallized from THF:MeOH (3 ml:0.5 ml) and diluted with Et_2O. Yield 0.423 g, 63%, m.p. 325–328 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 13.0 (br s, 1H), 9.80 (br s, 1H), 8.31 (d, J = 9.0 Hz, 1H), 8.20 (br d, J = 6.0 Hz, 2H), 8.12 (br s, 1H), 7.98 (br d, J = 6.0 Hz, 2H), 7.51 (br s, 1H), 7.27 (br s, 1H), 2.73 (m, 2H). Analysis: $C_{27}H_{32}N_4O_2 \cdot 1.60\ H_2O$ (C = 68.12; H = 7.25; N = 11.00).

*5.1.4. Method D (series 4): 2-(4-adamantanecarboxamido) phenyl)-N-(pyridin-2-yl)-1H-benzoimidazole-5-carboxamide (**4z**, Y = H)*

*5.1.4.1. 2-(4-Nitrophenyl)-1H-benzoimidazole-5-carboxylic acid (**20**, Y = H)*. A solution of 12.5 g (82.15 mmol) 3,4-diaminobenzoic acid (**10**) and 4-nitrobenzaldehyde (**19**) (12.5 g 82.7 mmol) in nitrobenzene (250 ml) was heated at 155–160 °C for 18 h. The reaction mixture was cooled at room temperature and diluted with diethyl ether (1 l). The yellow solids were collected by filtration, dissolved in hot DMF, treated with charcoal, and filtered. The filtrate was diluted with Et_2O to precipitate out the nitro acid **20** (11.45 g, 55%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.42 (m, 3H), 8.25 (br s, 1H), 7.94 (s, 1H), 7.88 (1H, d, J = 6.8 Hz, 1H), 7.71 (br s, 1H); ESI-MS (m/z): 284.2 (M + H) and 282.4 (M – H).

*5.1.4.2. 2-(4-Nitrophenyl)-1H-benzoimidazole-5-carboxylic acid pyridin-2-ylamide (**21**, R_1 = 2-pyridine, Y = H)*. A solution of nitro acid **20** (3.4 g, 12.0 mmol) and CDI (2.90 g, 18.0 mmol) in dry DMF (30 ml) was heated at 80 °C for 3 h. One equivalent of adamantaneamine hydrochloride (2.25 g, 12 mmol) was added twice at an interval of 2 h at room temperature, and then heated at 100 °C for 18 h. The reaction mixture was cooled to room temperature and diluted with

water. The yellow nitro-amide **21** was filtered and sequentially washed with water and MeOH. The yield was 2.80 g (65%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 13.79 (br s, 1H), 10.56 (br s, 1H), 8.46 (m, 2H), 8.29 (m, 2H), 8.13 (br s, 1H), 7.85 (br d, 1H), 7.78–7.71 (br m, 3H), 7.18 (br s, 1H), 7.05 (dt, J = 5.60 and 4.40 Hz, 1H). ESI-MS (*m*/*z*): 360.9 (M + H).

*5.1.4.3. 2-(4-Amino-phenyl)-1H-benzoimidazole-5-carboxylic acid pyridin-2-ylamide (**22**, R_1 = 2-pyridine, Y = H).* To a solution of 2.5 g (7.26 mmol) nitro pyridine-benzimidazole-carboxamide (**21**) in EtOH/DMF (9:1, 50 ml), 5% Pd/C (0.625 g) catalyst was added and the reaction mixture was heated under H_2 atmosphere at 90 °C for 3 h and at 70 °C for additional 3 h. The amine was freed from catalyst and EtOH was evaporated. The residue was diluted with water (100 ml) and product was filtered and air-dried. The amine **22** was re-crystallized from MeOH (yield: 0.80 g, 64%); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 12.71 (br s, 1H), 10.64 (br d, 1H), 8.38 (d, J = 4.0 Hz, 1H), 8.27 (br s, 1H), 8.21 (d, J = 6.40 Hz, 1H) 8.10 (br s, 1H), 7.89–7.81 (m, 3H), 7.60–7.48 (dd, J = 6.80 Hz, 1H), 7.15 (dd, J = 5.60 and 4.0 Hz, 1H), 6.68 (d, J = 6.80 Hz, 2H), 5.69–5.66 (br d, J = 12.0 Hz 2H); ESI-MS (*m*/*z*): 330 (M + H).

*5.1.4.4. 2-{4-[(Adamantane-1-carbonyl)-amino]-phenyl}-1H-benzoimidazole-5-carboxylic acid pyridin-2-ylamide (**4z**, Y = H).* To a solution of amine **22** (1.2 g, 3.65 mmol) in THF (15 ml) and pyridine (0.5 ml), adamantane-1-carbonyl chloride (1.09 g, 5.19 mmol) was added and stirred at 80 °C for 4 h. The resulting suspension was cooled and diluted with water. The amide **4z** was filtered and stirred with saturated $NaHCO_3$ solution for 2 h. The solid was filtered and washed with water and re-crystallized from MeOH (yield: 0.80 g, 45%); m.p. 328–330 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ 13.10 (br s, 1H), 10.73 (s, 1H), 9.38 (s, 1H), 8.40 (br d, J = 3.0 Hz), 8.29 (br s, 1H), 8.21 (d, J = 6.0 Hz, 1H), 8.14 (d, J = 9.0 Hz), 7.93–7.82 (m, 4 H), 7.64 (br d, J = 9.0 Hz, 1H), 7.17 (dt, J = 6.0 and 3.0 Hz, 1H), 2.04 (br s, 3H), 1.95 (s, 6H), 1.73 (s, 6H). ESI-MS (*m*/*z*): 492.6 (M + H). Analysis: $C_{30}H_{29}N_5O_2 \cdot 0.60\ H_2O$ (C = 71.43; H = 5.90; N = 14.13).

5.2. Biological assays

5.2.1. Materials

Goat anti-mouse IgE (GAME) and biotinylated GAME (b-GAME) were either prepared in-house or purchased from Bethyl Labs and The Binding Site, respectively. Murine and human recombinant IL-4 was obtained from Peprotech and Boehringer-Manheim, respectively. Anti-mouse and anti-human CD40 antibodies were obtained from Pharmingen. Female 8–12-week-old BALB/cByj mice were purchased from Jackson Laboratories and used in all mouse cell-based experiments. Spleen cells were obtained by sacrificing the mice by cervical dislocation, removal of the spleens, dispersing cells in a tissue grinder, filtering through nytex, washing twice with phosphate-buffered saline (PBS), and culturing (5×10^6 lymphocytes per ml) in DMEM/10% fetal bovine serum (FBS) at 37 °C, 10% CO_2.

5.2.2. Ex vivo IgE assay

The ex vivo Ig response assay involves in vivo antigen priming and measures secondary antibody responses in vitro. The basic protocol has been thoroughly documented and optimized for a range of parameters including: antigen dose for priming and time span following priming; number of cells cultured in vitro; antigen concentrations for eliciting secondary IgE (and other Igs) response in vitro; FBS batch that will permit optimal IgE response in vitro; the importance of primed CD4-positive T cells and hapten-specific B cells; and specificity of the ELISA assay for IgE [22]. Female BALB/cByj mice were immunized intraperitoneally (i.p.) with 10 μg dinitrophenol-conjugated keyhole limpet hemocyanin (DNP_6-KLH) adsorbed onto 4 mg alum and sacrificed after 14–20 days. Spleens were removed and homogenized in a tissue grinder, washed twice, and spleen cell cultures (3×10^6 lymphocytes per ml) established in 96-well round-bottom plates in the presence or absence of DNP_6-KLH (10 ng/ml). Test compounds were added to spleen cell cultures immediately prior to addition of antigen. Cultures were prepared in quadruplicate and incubated for 7–12 days.

5.2.3. In vitro IgE assay

Spleen cells were obtained from naïve female BALB/c mice, and T cells were depleted prior to culture by incubating spleen cells first with a cocktail of anti-Thy1 ascites (10%), anti-CD4 Ab (0.5 μg/ml) and anti-CD8 Ab (0.5 μg/ml), followed by guinea pig complement (agarose adsorbed). Cells were cultured at 3×10^6 per ml in quadruplicate. Culture conditions were followed as described in the ex vivo protocol except that instead of antigen, Ig production was stimulated by IL-4 (10 ng/ml) and anti-CD40 antibody (100 ng/ml). After 5–7 days of culture, 2 μl of supernatant was removed for quantifying by ELISA. The ELISA protocol essentially follows as described in the ex vivo protocol except that since the IgE is not produced in response to specific antigen, the polyclonal IgE is retained on the plates by coating them with GAME (1 μg/ml).

5.2.4. Oral bioavailability

Female BALB/c mice (five mice per compound) were administered a single dose of 40 mg/kg suspended at 10 mg/ml in 0.2 M citric acid (pH 4). After 1 h mice were bled and the samples pooled. Serum was obtained by centrifuging twice to eliminate cells, adding an equal volume of acetonitrile, vortexing, centrifuging, and collecting the eluate. The solution phase was dried in a vacufuge (Eppendorf), sonicated in mobile phase (0.1% TFA in water/acetonitrile, 30:70), centrifuged, and analyzed by reverse-phase HPLC. Sample compounds were quantified by comparison to a standard curve.

5.2.5. IgE response in human cells

Freshly drawn human peripheral blood was diluted 1:1 with sterile PBS and layered over 10 ml of Histopaque. The tubes were centrifuged at 1800 rpm (400 × *g*) for 30 min at room temperature. The plasma layer (yellow) was aspirated and the white cells collected by removing the interphase. This layer was transferred to a new 50 ml conical tube containing 20 ml of sterile PBS. White blood cells were counted, centrifuged at 1000 rpm (250 × *g*) for 10 min, and re-suspended in 15 ml of sterile PBS. Human PBL cultures (5×10^5 cells per ml) were established in quadruplicate in the presence of human IL-4 (Boehringer-Manheim) and anti-human CD40 Ab (Pharmingen), and incubated at 37 °C; 10% CO_2 for 10–14 days before harvesting supernatants for the ELISA.

5.2.6. In vivo IgE response

Female BALB/c mice (5–10 per group) were administered 250 rads (except control group) followed by immunizing with 2 μg KLH in alum (4 mg) i.p. 2 h later [59,60]. **1mm** was injected i.p. on 3 consecutive days starting 6 days later. On day 2 of the drug injections, 2 μg of DNP_6-KLH with alum (4 mg) was injected i.p. Periorbital bleeds were obtained 21 days following DNP_6-KLH challenge and the serum was processed for quantification of antigen-specific antibody by ELISA.

5.2.7. Ig ELISA

To detect DNP-specific antibodies in cultures of mouse spleen cells, ELISA plates are prepared by coating with specific antigen (DNP-OVA) overnight. After washing and blocking the plates with 200 μl bovine serum albumin (BSA) in PBS, an aliquot (1:4 dilution in PBS with 1% BSA 0.1% azide 0.5% Tween 20) of each culture supernatant was transferred directly to the ELISA plates and incubated overnight in a humidified box at 4 °C. IgE was quantified following successive 90 min incubations with biotinylated GAME (prepared in-house), alkaline phosphatase-streptavidin (Zymed), and 100 μl of phenolphthalein monophosphate (PPMP, DCHA salt, 40 mg/ml). Absorption was measured at 540λ. The level of detection for IgE is about 200–400 pg/ml and there is less than 0.001% cross-reactivity with any other Ig isotype in the ELISA for IgE [56].

To measure DNP-specific antibodies from serum, ELISA plates were prepared as with the ex vivo assay by coating with 50 μl DNP_{15}-conjugated ovalbumin (DNP_{15}-OVA, 5 μg/ml). Serum samples were diluted in saline and tested in triplicate as follows: for antigen-specific IgE and IgG2a, dilute 1:200, 1:400, 1:800, and 1:1600; for IgG1, dilute 1:16,000, 1:32,000, 1:64,000, and 1:128,000. Plates were stored overnight at 4 °C in a humidified chamber, washed, and treated as described for the ex vivo ELISA. Antigen-specific IgG1 and IgG2a were measured similarly except that biotinylated (b)-GAMG1 and b-GAMG2a (Southern Biotechnology Associates) were substituted for b-GAME. Quantification of each isotype was determined by comparison to a standard curve. In control animals, serum DNP-specific IgE, IgG1, and IgG2a were typically 20, 2000, and 10 μg/ml, respectively, on day 21 after antigen challenge.

Human IgE was quantified from PBL culture supernatants on round-bottom plates that were coated overnight with 1 μg/ml GAHE (Sigma) in PBS. The plates were washed and blocked with 200 μl per well of 1% BSA in PBS/azide. After washing, a solution of BSA/PBS/Tween/azide was added to all wells, and 15 μl of the PBL culture supernatant was added to the corresponding plates. DE-52-purified IgE from a plasmacytoma (PS) was used as standard. Samples and standards were allowed to bind overnight at 4 °C. The plates were washed and incubated with 50 μl of 1 μg/ml biotin anti-human IgE (the binding site) for 1.5 h at 37 °C. After washing the plates, 50 μl of the 1:1000 dilution of AP-streptavidin was added and incubated an additional 1.5 h. After washing, 100 μl of a 40 mg/ml working solution of 26 × PPMP was added and the absorbance read at 540λ.

5.2.8. Cytokine assays

Drug activity was tested in two distinct assay systems utilizing spleen cells from female BALB/c mice. For the in vitro assay, T cells were isolated using Pan-T (CD3ε) magnetic bead kit (Miltenyi Biotec) and cultured for 48 h in the presence of ConA (5 μg/ml) in the presence and absence of drug. Culture supernatants were tested for IL-4 and IL-5 content using fluorescent beads from a cytokine detection kit (Upstate) and analyzed by a Luminex 100 flow analyzer. IL-4 and IL-5 were quantified by comparison to standard curves. The results obtained with this method were found to agree well with ELISA assays using reagents and protocols obtained from BD-Pharmingen (not shown).

For the ex vivo cytokine response assay, spleens were removed from mice that had been sensitized to DNP_6-KLH, as described for the ex vivo IgE response assay. Two weeks following sensitization, mice were sacrificed and spleen cell cultures established in the presence of DNP_6-KLH. After 4 days, ConA (5 mg/ml) was added to cultures and the cells incubated for an additional 4 h. Cytokine levels in the culture supernatants were quantified by ELISA using reagents and protocols supplied by Pharmingen. Control responses were determined from cultures that contained no drug.

5.2.9. Murine CD23 expression

Spleen cells (10^6 per ml) were dispersed from the spleens of non-sensitized BALB/c mice and cultured for 24 or 48 h. Expression of CD23 was stimulated with IL-4 (10 ng/ml) and anti-CD40 antibody (100 ng/ml), while parallel cultures contained the 2-phenyl benzimidazole compound. Flow cytometry was carried out on a FACScan (Becton-Dickinson) using FITC-conjugated monoclonal anti-CD23 antibody (B3B4, Pharmingen).

Acknowledgements

We thank Dr. Sunil Kumar KC for critical review of the manuscript.

References

[1] G.J. Rodrigo, C. Rodrigo, J.B. Hall, Chest 125 (2004) 1081–1102.
[2] H.W. Kelly, Pharmacotherapy 17 (1997) 13S–21S.
[3] F.D. Martinez, Respir. Res. 2 (2001) 129–132.
[4] S. Romagnani, J. Allergy Clin. Immunol. 113 (2004) 395–400.
[5] J.J. McIntire, D.T. Umetsu, R.H. DeKruyff, Springer Semin. Immunopathol. 25 (2004) 335–348.
[6] D.T. Umetsu, Nat. Med. 10 (2004) 232–234.
[7] C. Walker, E. Bode, L. Boer, T.T. Hansel, K. Blaser, J.C. Virchow Jr., Am. Rev. Respir. Dis. 146 (1992) 109–115.
[8] D.S. Robinson, Q. Hamid, S. Ying, A. Tsicopoulos, J. Barkans, A.M. Bentley, et al., N. Engl, J. Med. 326 (1992) 298–304.
[9] S. Matsushita, M.L. Richards, D.H. Katz, in: E.B. Weiss, M. Stein (Eds.), Bronchial Asthma, Mechanisms and Therapeutics, third ed, Little, Brown and Company, Boston, 1993, pp. 57–67.
[10] H.J. Gould, B.J. Sutton, A.J. Beavil, R.L. Beavil, N. McCloskey, H.A. Coker, et al., Annu. Rev. Immunol. 21 (2003) 579–628.
[11] J.G. Scadding, K.M. Moser, in: E.B. Weiss, M. Stein (Eds.), Bronchial Asthma, Mechanisms and Therapeutics, third ed, Little, Brown and Company, Boston, 1993, pp. 3–14.
[12] B.A. Berman, J. Allergy Clin. Immunol. 81 (1988) 980–984.
[13] P.J. Barnes, K.F. Chung, C.P. Page, Pharmacol. Rev. 50 (1998) 515–596.
[14] C. Taube, A. Dakhama, E.W. Gelfand, Int. Arch. Allergy Immunol. 135 (2004) 173–186.
[15] H. Milgrom, R.B. Fick Jr., J.Q. Su, J.D. Reimann, R.K. Bush, M.L. Watrous, et al., N. Engl, J. Med. 341 (1999) 1966–1973.
[16] M. Haak-Frendscho, K. Robbins, R. Lyon, R. Shields, J. Hooley, M. Schoenhoff, et al., Immunology 82 (1994) 306–313.
[17] H. Lin, K.M. Boesel, D.T. Griffith, C. Prussin, B. Foster, F.A. Romero, et al., J. Allergy Clin. Immunol. 113 (2004) 297–302.
[18] P. Yu, M. Kosco-Vilbois, M.L. Richards, G. Kohler, M.C. Lamers, Nature 369 (1994) 753–756.
[19] L. Flores-Romo, J. Shields, Y. Humbert, P. Graber, J.P. Aubry, J.F. Gauchat, et al., Science 261 (1993) 1038–1041.
[20] A. Saxon, M. Kurbe-Leamer, K. Behle, E.E. Max, K. Zhang, J. Immunol. 147 (1991) 4000–4006.
[21] A. Stief, G. Texido, G. Sansig, H. Eibel, G. Le Gros, H. van der Putten, J. Immunol. 152 (1994) 3378–3390.
[22] G. Texido, H. Eibel, G. Le Gros, H. van der Putten, J. Immunol. 153 (1994) 3028–3042.
[23] A. Haczku, K. Takeda, E. Hamelmann, A. Oshiba, J. Loader, A. Joetham, et al., Am. J. Respir. Crit. Care Med. 156 (1997) 1945–1955.
[24] R.M. Ten, M.J. McKinstry, G.D. Bren, C.V. Paya, J. Allergy Clin. Immunol. 104 (1999) 376–387.
[25] M.R. Kehry, L.C. Yamashita, Proc. Natl. Acad. Sci. USA 86 (1989) 7556–7560.
[26] U. Pirron, T. Schlunck, J.C. Prinz, E.P. Rieber, Eur. J. Immunol. 20 (1990) 1547–1551.
[27] E.A. Clark, J.A. Ledbetter, Nature 367 (1994) 425–428.
[28] L.B. Bacharier, R.S. Geha, J. Allergy Clin. Immunol. 105 (2000) S547–S558.
[29] G. Grunig, M. Warnock, A.E. Wakil, R. Venkayya, F. Brombacher, D.M. Rennick, et al., Science 282 (1998) 2261–2263.
[30] N. Miyahara, K. Takeda, T. Kodama, A. Joetham, C. Taube, J.W. Park, et al., J. Immunol. 172 (2004) 2549–2558.
[31] G. Brusselle, J. Kips, G. Joos, H. Bluethmann, R. Pauwels, Am. J. Respir. Cell Mol. Biol. 12 (1995) 254–259.
[32] D.B. Corry, H.G. Folkesson, M.L. Warnock, D.J. Erle, M.A. Matthay, J.P. Wiener-Kronish, et al., J. Exp. Med. 183 (1996) 109–117.
[33] D.C. Webb, A.N. McKenzie, A.M. Koskinen, M. Yang, J. Mattes, P.S. Foster, J. Immunol. 165 (2000) 108–113.
[34] G.J. Gleich, J. Allergy Clin. Immunol. 85 (1990) 422–436.
[35] M. Wills-Karp, C.L. Karp, Science 305 (2004) 1726–1729.
[36] D.Y. Wang, P. Clement, Am. J. Rhinol. 14 (2000) 325–333.
[37] J.M. Wang, A. Rambaldi, A. Biondi, Z.G. Chen, C.J. Sanderson, A. Mantovani, Eur. J. Immunol. 19 (1989) 701–705.
[38] H.D. Campbell, W.Q. Tucker, Y. Hort, M.E. Martinson, G. Mayo, E.J. Clutterbuck, et al., Proc. Natl. Acad. Sci. USA 84 (1987) 6629–6633.
[39] B. Gregory, A. Kirchem, S. Phipps, P. Gevaert, C. Pridgeon, S.M. Rankin, et al., J. Immunol. 170 (2003) 5359–5366.
[40] J. Drazen, Am. J. Respir. Crit. Care Med. 157 (1998) S233–S237 (discussion S247–S248).
[41] M. Hasegawa, K. Takenouchi, K. Takahashi, T. Takeuchi, K. Komoriya, Y. Uejima, et al., J. Med. Chem. 40 (1997) 395–407.
[42] Y. Tada, I. Yamawaki, S. Ueda, H. Matsumoto, N. Matsuura, M. Yasumoto, et al., J. Med. Chem. 41 (1998) 3330–3336.
[43] G. Hochhaus, L. Brookman, H. Fox, C. Johnson, J. Matthews, S. Ren, Y. Deniz, Curr. Med. Res. Opin. 19 (2003) 491–498.
[44] Y. Yanagihara, M. Kiniwa, K. Ikizawa, T. Shida, N. Matsuura, A. Koda, Jpn. J. Pharmacol. 61 (1993) 31–39.
[45] Y. Yanagihara, M. Kiniwa, K. Ikizawa, H. Yamaya, T. Shida, N. Matsuura, et al., Jpn. J. Pharmacol. 61 (1993) 23–30.
[46] P.D. Mehlhop, M. van de Rijn, A.B. Goldberg, J.P. Brewer, V.P. Kurup, T.R. Martin, et al., Proc. Natl. Acad. Sci. USA 94 (1997) 1344–1349.
[47] M.J. Leckie, A. ten Brinke, J. Khan, Z. Diamant, B.J. O'Connor, C.M. Walls, et al., Lancet 356 (2000) 2144–2148.
[48] C. Buttner, A. Lun, T. Splettstoesser, G. Kunkel, H. Renz, Eur. Respir. J. 21 (2003) 799–803.
[49] S.A. Bryan, B.J. O'Connor, S. Matti, M.J. Leckie, V. Kanabar, J. Khan, et al., Lancet 356 (2000) 2149–2153.
[50] H. Renz, K. Enssle, L. Lauffer, R. Kurrle, E.W. Gelfand, Int. Arch. Allergy Immunol. 106 (1995) 46–54.
[51] T.A. Sato, M.B. Widmer, F.D. Finkelman, H. Madani, C.A. Jacobs, K.H. Grabstein, et al., J. Immunol. 150 (1993) 2123–2717.
[52] L.J. Rosenwasser, W.W. Busse, R.G. Lizambri, T.A. Olejnik, M.C. Totoritis, J. Allergy Clin. Immunol. 112 (2003) 563–570.
[53] F.E. Hargreave, J. Dolovich, M.T. Newhouse, J. Allergy Clin. Immunol. 85 (1990) 1098–1111.
[54] E.A. Mitchell, J.M. Bland, J.M. Thompson, Thorax 49 (1994) 33–36.
[55] M.L. Richards, S.C. Lio, A. Sinha, K.K. Tieu, J.C. Sircar, J. Med. Chem. 47 (2004) 6451–6454.
[56] J.F. Marcelletti, D.H. Katz, Cell. Immunol. 135 (1991) 471–489.
[57] N. Chiorazzi, D.A. Fox, D.H. Katz, J. Immunol. 117 (1976) 1629–1637.
[58] D.A. Fox, N. Chiorazzi, D.H. Katz, J. Immunol. 117 (1976) 1622–1628.
[59] K. Zhang, E.A. Clark, A. Saxon, J. Immunol. 146 (1991) 1836–1842.
[60] R.L. Coffman, J. Ohara, M.W. Bond, J. Carty, A. Zlotnik, W.E. Paul, J. Immunol. 136 (1986) 4341–4538.
[61] M.L. Richards, D.H. Katz, Crit. Rev. Immunol. 11 (1991) 65–86.
[62] J. Punnonen, G. Aversa, B.G. Cocks, J.E. de Vries, Allergy 49 (1994) 576–586.
[63] M.L. Richards, D.H. Katz, J. Immunol. 158 (1997) 263–272.