Pergamon

Bioorganic & Medicinal Chemistry 9 (2001) 1793–1805

BIOORGANIC & MEDICINAL CHEMISTRY

Disease Activated Drugs: A New Concept for the Treatment of Asthma

Brigitte Charpiot,[a,*] Francis Bitsch,[a] Karl-Heinz Buchheit,[a] Pascal Channez,[b] Lazzaro Mazzoni,[a] Thomas Mueller,[a] Isabelle Vachier[b] and Reto Naef[a]

[a]*Research, Novartis Pharma AG CH-4002 Basle, Switzerland*
[b]*CHU Montpellier, Hôpital Arnaud de Villeneuve, 34295 Montpellier, France*

Received 12 September 2000; accepted 14 March 2001

Abstract—Disease activated drugs (DAD) are pro-drugs of one active principle or combinations of two drugs, which have a proven efficacy for the treatment of the target disease. In opposition to pro-drugs, DAD are activated in inflamed but not normal tissues. Due to the disease specific activation, the amount of locally released drug(s) should be related directly to the severity of the inflammation. To test this concept in asthma a PDE4 inhibitor, an isoquinoline derivative, was chemically derivatized into pro-drugs or combined with corticosteroids. These new compounds were more readily cleaved into active PDE4 inhibitor, in bronchoalveolar lavage fluid (BALF) from Brown-Norway rats with lung inflammation than in BALF from rats without airway inflammation. The DAD concept (local selective release and improved therapeutic window) was validated in vivo using the inhibition of methacholine induced bronchoconstriction in guinea pigs with or without ozone induced lung inflammation. An example of DAD hydrolysis (isoquinoline-dexamethasone) was also examined in BALF from asthmatics and healthy volunteers.

Introduction

The concept of disease activated drugs (DAD) is new, and theoretically, could be applied to many chronic inflammatory diseases. DAD are derivatized drugs or combinations of two covalently linked drugs. These derivatized drugs or drug combinations, per se inactive, are to be activated locally by a disease specific mechanism (e.g., proteolytic enzymes, free radical mediators, alteration in local pH or redox potential) specifically in the inflamed tissues (Fig. 1). This approach could potentially present several advantages: reduced side effects (since active drugs are released only in the inflamed tissues) and thus improve therapeutic window, possibility of modulating the pharmacokinetics of the single drugs, potential additive or even synergistic effects by combination of compounds with different mechanism of action.

DAD might be applied in various chronic diseases with episodic progression but it seems that chronic inflammatory conditions (e.g., asthma) would be the ideal target to test the feasibility of such a new concept. The use of phosphodiesterase type 4 (PDE4) inhibitors and their combination with corticosteroids appears therefore as an attractive example for the evaluation of the DAD concept.

Cyclic 3′,5′-AMP (c-AMP) exerts its action in the cell by binding to and activating protein kinase A. This family of enzymes elicits the phosphorylation of specific target proteins, leading to functional changes within the cell. Changes in the c-AMP signalling system have been noted in a number of diseases, such as asthma,

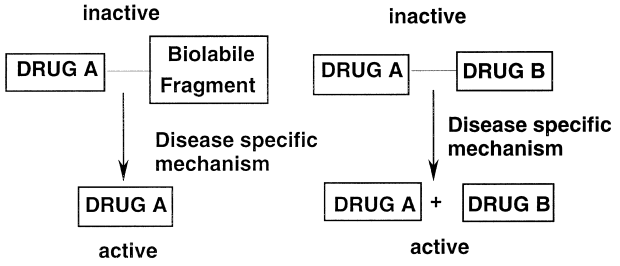


Figure 1. DAD concept.

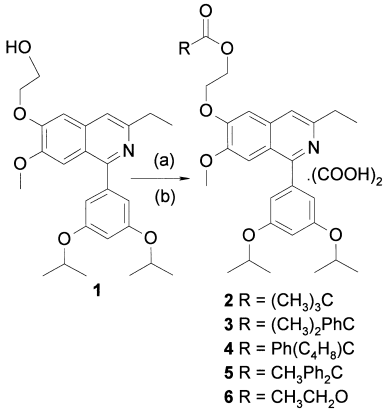
*Corresponding author. Tel.: +41-61-324-6208; fax: +41-61-324-7875; e-mail: brigitte.charpiot@pharma.novatis.com

0968-0896/01/$ - see front matter
PII: S0968-0896(01)00077-3

inflammation and atopy. The only known way cells inactivate c-AMP is by hydrolysing it to 5′-AMP. This is achieved through the action of a family of enzymes called cyclic AMP phosphodiesterases (EC 3.1.4.17). PDE4 enzymes, a subfamily of the phosphodiesterases, are able to hydrolyse specifically c-AMP and have received considerable attention as molecular targets for novel anti-asthmatic agents.[1a–c] PDE4 inhibitors have been shown to impair the influx and activation of neutrophils, eosinophils, lymphocytes and other cells of the immune system.[2] In the treatment of asthma, the application by inhalation of a DAD containing a PDE4 inhibitor could further reduce the side-effects due to the ubiquitousness of PDE activity, a common problem of this inhibitor class.[3] In addition the therapeutic association of a PDE4 inhibitor and a corticosteroid (dexamethasone, budesonide) in a DAD could possibly potentiate the effect of the single components. For proof of concept, compound **1**, ethanol, 2-[1-(3,5-bis(1-methyl-ethoxy)-phenyl)-3-ethyl-7-methoxy-6-isoquinolinyl] oxy], a selective, potent inhibitor of PDE4 enzyme with high anti-inflammatory activity,[4] was selected as parent component. Derivatives of **1** and combinations with corticosteroids (dexamethasone and budesonide) were synthesised as model molecules for the validation of the DAD concept. Dexamethasone and budesonide were chosen as corticosteroids since they were tested as equipotent with compound **1** in our in vivo model of airway inflammation.

The design of the linker was subjected to the following considerations: the linker should not generate a toxic entity during the hydrolysis process, it should not increase significantly the synthesis cost of the final molecule and finally should allow an almost simultaneous release of both active compounds. Thus, we focused on a carbonate linker (for PDE4 inhibitor–corticosteroid hybrid molecules) as carbon dioxide release is of no consequence for the lungs.

In the first step, these molecules were used to test the feasibility of the concept in vivo and in vitro. A next step, as part of a mechanistic approach, would aim at the determination of the enzyme(s) involved in the cleavage process.



Scheme 1. Reagents and conditions used: (a) 1.2 equiv RCOCl, 1.2 equiv NEt_3;(b) oxalic acid in ether (except for **5**).

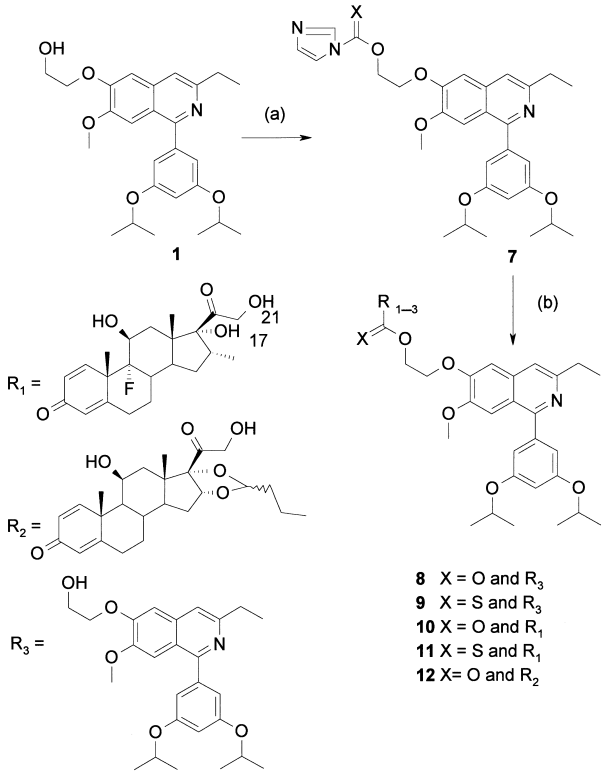
Chemistry

The general route for synthesising the acyl derivatives of the PDE4 inhibitor, **1**, is illustrated in Scheme 1. Coupling via the free hydroxyl function of **1** with the appropriate acid chloride occurred in presence of a base, usually dried triethylamine and gave compounds **2**–**5** in moderate to good yields.

The carbonate derivative **6** was obtained by reacting ethyl chloroformate and **1**. Formation of the mono oxalic salts facilitated the purification of some compounds or improved their physicochemical properties.

The use of 1,1′-carbonyldiimidazole (respectively 1,1′-thiocarbonyldiimidazole for the synthesis of **9** and **11**) turned out to be necessary for the synthesis of the homodimer of **1**, **8**, and the combination products of **1** with dexamethasone, **10**, and **1** with budesonide, **12**, (Scheme 2). Other coupling reagents, for example phosgene, ethyl chloroformate, were unsuccessful. The intermediate **7** is a stable compound and can be isolated.

The acylation of dexamethasone was only possible in position 21. Although several dexamethasone-17 alkyl carbonate derivatives are described in the literature,[5] none of these methods (enzymatic alkoxycarbonylation from vinyl carbonate or methyl carbonate catalyzed by *Candida antartica lipase*,[6a–c] synthesis of dexamethasone-17,21-dialkylorthocarbonate and successive



Scheme 2. Reagents and conditions used: (a) 1 equiv 1,1′-carbodiimidazole (1,1′-thiocarbonyldiimidazole, respectively), 4-dimethylamino pyridine (4 equiv); (b) 1 equiv dexamethasone (budesonide or **1**).

rearrangement,[5] silylation of the 21-hydroxyl and direct acylation of the 17-position or reaction of the 17-hydroxyl with alkyl halide and silver carbonate[7]) led to the 17-acylated desired product. The budesonide analogue, **12**, was synthesised following the preceding method.

Results and Discussion

DAD, as previously mentioned, should be per se inactive molecules. In our context, they should not inhibit PDE4 enzyme and also, to reduce the potential for cardio-vascular side-effects, should be inactive as PDE3 inhibitors.

Phosphodiesterase enzymatic assays

The compounds synthesised are far less potent inhibitors of PDE4 than **1** and are mostly inactive against PDE3 (Table 1). The compounds were tested against both PDE4 enzymes, isolated from human neutrophil homogenates, and cloned rat PDE4B enzyme. Since human PDE4B, a subtype of the PDE4 family, seems to be the dominant PDE4 isogene in neutrophils[8] and is 92% homologous to rat PDE4B,[9] results from both preparations should be comparable. Indeed compounds **1**, **6** and **9,** which were tested for PDE4 and PDE4B inhibition, showed similar activities in both assays.

Hydrolysis studies in rat bronchoalveolar lavage fluids (BALF)

Efflux of eosinophils into the airway lumen, following inhalation of the antigen, is a distinctive feature of asthma. Analogous to the effects in asthmatics, an accumulation of inflammatory cells (neutrophils, eosinophils and mononuclear cells) and a release of mediators in the airways occur when actively sensitised Brown Norway (BN) rats inhale modest amounts of allergen. Such animal challenge models can be used to suggest potential clinical efficacy of anti-asthma drugs.[10] Based on this, we aimed at developing an in vitro assay to test our working hypothesis that DAD, administered by inhalation, are cleaved in the lung of allergic animals and remain uncleaved in normal animals. The in vitro assay which seems to be adequate for this purpose is the hydrolysis study of the different compounds in bronchoalveolar lavage fluids (BALF) of BN rats sensitised and challenged with ovalbumin (BALF+) compared to the BALF of sensitised, but non-challenged BN rats (BALF−). Compound **13**,[11] (Fig. 2), which has a chemical structure similar to **1**, was used as an internal reference for the relative quantification of compounds in the BALF samples.

Firstly, we investigated whether DAD **2**–**5** (ester derivatives of **1**) could be hydrolysed, that is activated, in vitro in the two different BALF.

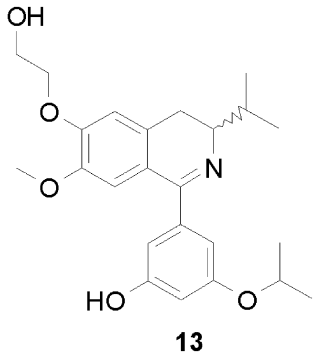
Data obtained with the esters was promising (Fig. 3). After 1 h, hydrolysis of **2** yielded 38% of **1** in BALF+ and 6% in BALF−. The same pattern of hydrolysis was also observed with other esters. A fine tuning of the hydrolysis rate in BALF, while the selectivity is still conserved, was possible by the introduction of sterically hindered residues on the ester side (compound **2** compared to **3**–**5**).

The carbonate, **6**, exhibited a similar pattern of hydrolysis as compound **2** (Fig. 3). Carbamate derivatives of **1** (*tert*-butyl, benzyl) were stable during the 24 h incubation in BALF+ and BALF− (data not shown). We then looked at DAD **8**–**12**, which are inactive hybrid molecules of two per se active compounds. In general, a dimer of **1**, **8**, and the hybrids dexamethasone-**1**, **10**, and budesonide-**1**, **12**, were cleaved more slowly than the esters **2**–**4**, nevertheless **1** was still formed more rapidly in BALF+ than in BALF− (Fig. 4). Remarkably, compound **8**, which allows free access to the hydrolizable carbonyl function was cleaved less efficiently than compound **10**, which is more sterically hindered. This finding indicates that the hydrolysis rate is not entirely driven by steric hindrance but probably also by enzyme substrate recognition. Surprisingly, the thio-analogues of **8**, **9** (data not shown), and of **10**, **11**, remained uncleaved after 24 h incubation in both BALF. This result reinforces the hypothesis that an enzymatic reaction is involved in the hydrolysis.

Table 1. Inhibition of phosphodiesterase isoenzymes[a]

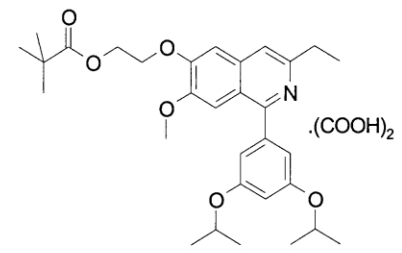
Compounds	PDE3	PDE4 (human neutrophils)	PDE4B (rat)
1	0.48±0.15 (3)	0.0036±0.0005 (10)	0.0042±0.0001 (130)
2	>100	3.71	—[b]
3	—	2.3	—
4	—	60.2	—
5	—	>100	—
6	>100	1.7	2.04
8	—	0.14 (2)	—
9	0.076 (2)	0.17 (2)	0.29 (2)
11	>10	—	0.26 (2)
12	—	—	0.7 (2)

[a]Data represent mean IC_{50} values μ±SEM (μM) with the number of observations shown in brackets.
[b]Means not determined.

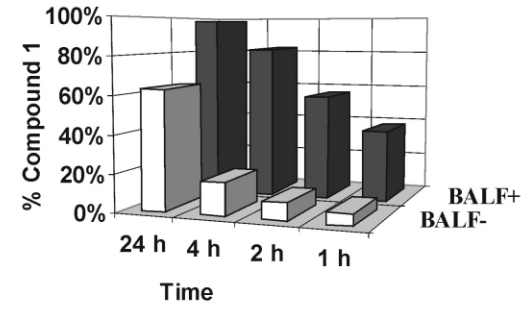


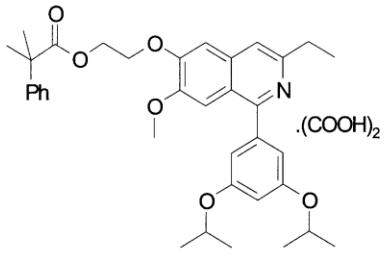
13

Figure 2. Structure of compound **13**.

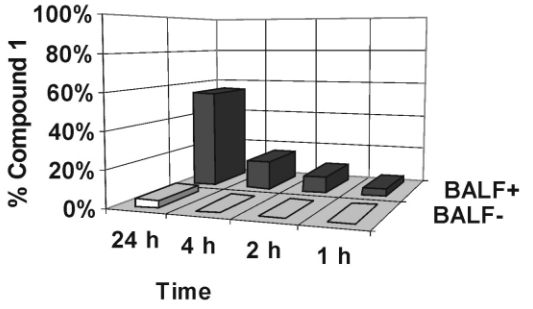


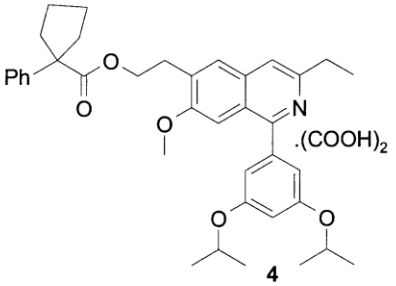
2



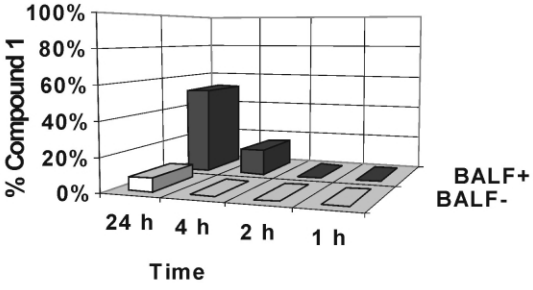


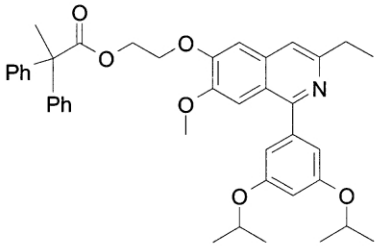
3



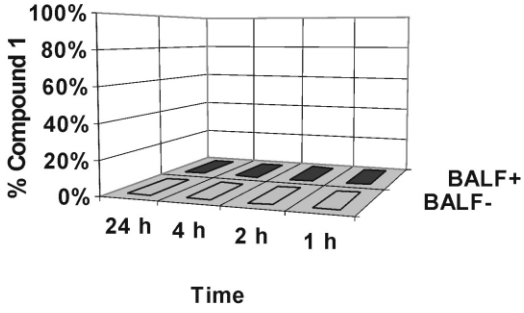


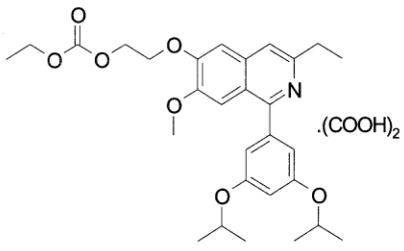
4





5





6

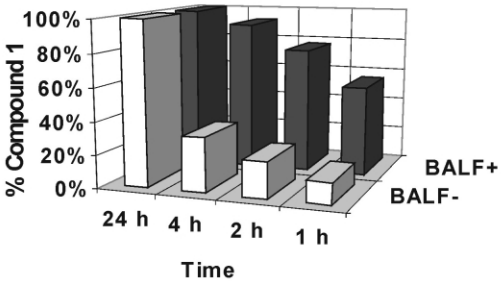
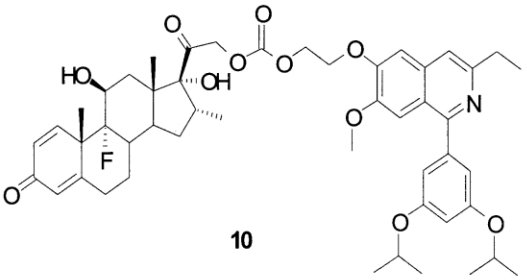
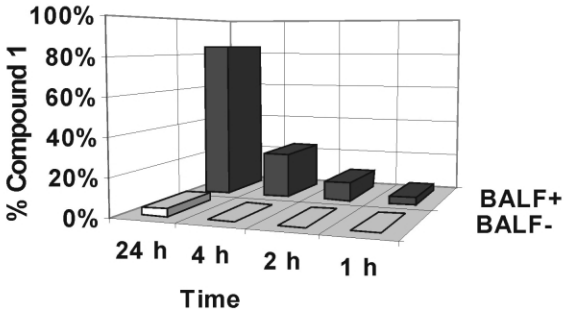
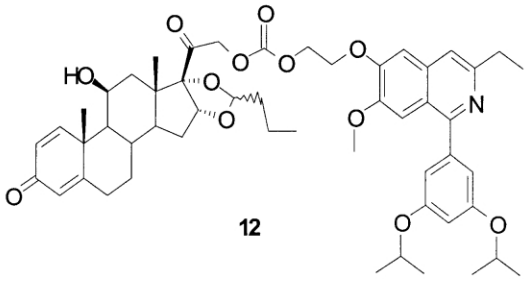
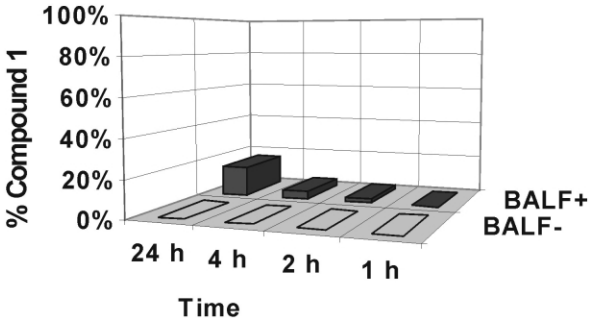


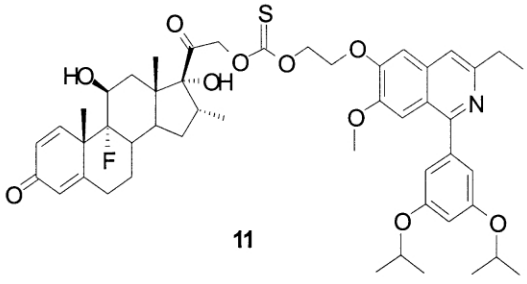
Figure 3. Hydrolysis of DAD esters in BALF from sensitized-challenged Brown Norway rats (BALF+) and BALF from sensitized-non-challenged Brown Norway rats (BALF−). Shown are the means of two aliquots.

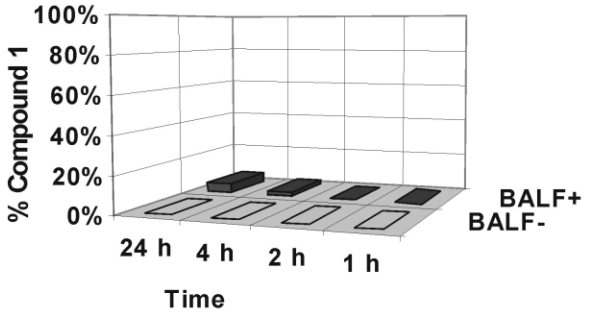


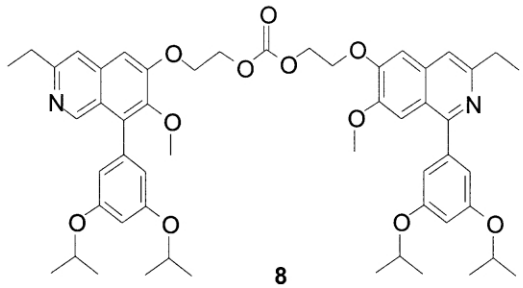












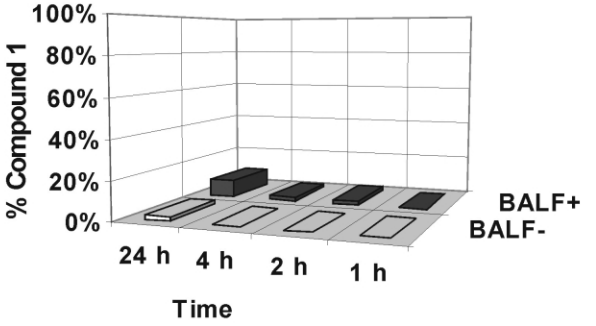


Figure 4. Hydrolysis of DAD carbonates in BALF from sensitized-challenged Brown Norway rats (BALF+) and BALF from sensitized-non-challenged Brown Norway rats (BALF−). Shown are the means of two aliquots.

Bronchoconstriction in ozone- and non-ozone treated guinea pigs

In a wide variety of species, including man,[12] rats [13] and guinea pigs,[14] inhalation of ozone induces an inflammatory process in the lung, accompanied by increased contractile reaction of the airways to exogenous and endogenous spasmogens such as methacholine. As a result, the bronchoconstrictor effect to a given dose of, for example methacholine, is markedly enhanced in ozone- as compared to non-ozone treated guinea pigs.

Since PDE4 inhibitors, like **1**, have the potential to inhibit bronchoconstriction,[15a–d] the assessment of the anti-bronchoconstrictor effect of DAD in ozone- and non-ozone-treated guinea pigs offers the possibility of (1) determining the degree of hydrolysis of these compounds in vivo and (2) proving the concept of DAD in animals. If a DAD is cleaved to the active principle **1**, by enzymes released in the ozone-induced airway inflammatory process, an inhibition of the bronchoconstriction provoked by methacholine is to be expected. This anti-bronchoconstrictor effect can fully be ascribed to the release of **1**, if the DAD molecule does not inhibit bronchoconstriction in non-ozone-treated guinea pigs, where no inflammation process is present. However, this fully depends on the assumption that for **1**, the released active principle, an anti-bronchoconstrictor effect can be observed in the chosen animal model. Our data demonstrate a dose-dependent anti-bronchoconstrictor effect of **1**, which can be measured both in ozone- and non-ozone-treated guinea pigs. In ozone-pretreated animals, **1** inhibited methacholine-induced bronchoconstriction by 73% (maximal effect, obtained 30 min after administration) at a dose of 30 μg/kg given intratracheally. In guinea pigs, which were not treated with ozone, the maximal effect of **1** was identical (78% inhibition, 30 min after administration) (Fig. 5).

In contrast to **1**, compounds **2** and **6**, which were readily hydrolyzed in BN rat BALF+, also inhibited methacholine-induced bronchoconstriction in ozone-treated guinea pigs but not in guinea pigs which were not treated with ozone (Fig. 5). The doses that were necessary

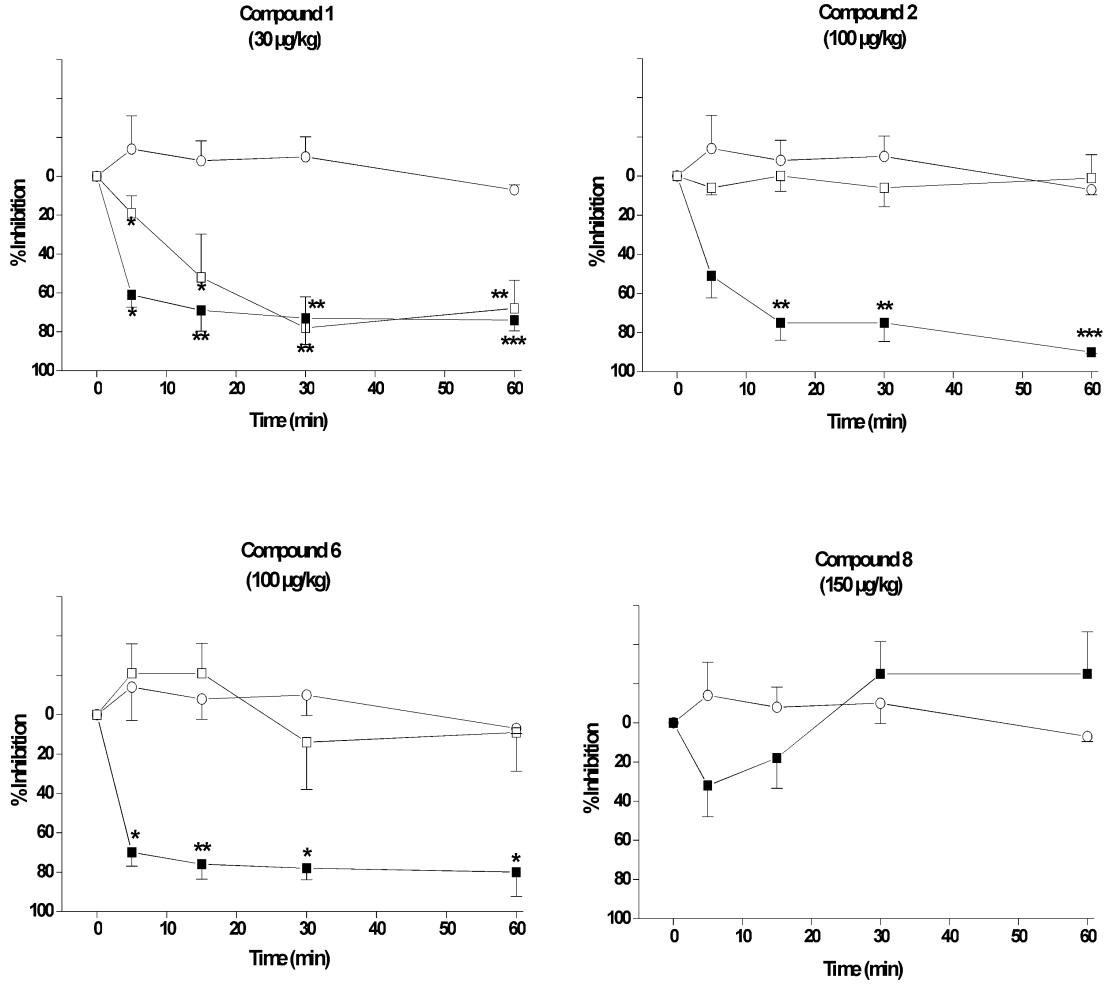


Figure 5. Time course of the effect of compounds **1**, **2**, **6**, **8** and their vehicle (○) on methacholine induced bronchoconstriction in ozone-pretreated (■) and non-ozone-pretreated (□) guinea pigs. Shown is the inhibition of the bronchoconstrictor response in % of the response to methacholine in the absence of test compounds as mean ± SEM in three to five animals. The compounds were given it at timepoint 0. Significant differences between effects of the vehicle and the test compounds are indicated: $*p<0.05$; $**p<0.01$; $***p<0.001$ (Student's *t*-test).

for a comparable degree of inhibition were marginally higher than those used in the case of **1**. The lack of inhibition obtained in healthy guinea pigs with these two compounds clearly indicates their inability to relax airway smooth muscle when they are not cleaved into **1**. This observation is consistent with their low inhibitory activity against PDE3 ($IC_{50} > 100$ μM) or PDE4 (Table 1) as bronchodilatation is often associated with inhibition of PDE3 or/and PDE4 activity. In addition it shows that the molecules were not hydrolysed in non-ozone-treated guinea pigs. As a consequence, it can be concluded that the inhibitory effect obtained in ozone-treated animals is due to the release of **1** in the lung. Even though higher doses are required as for **1** itself, the difference might be explained by the kinetics of the enzymatic hydrolysis and the pharmacokinetics of **1** and DAD respectively in guinea pigs. For example, if the rate of hydrolysis of compounds **2** and **6** to **1** is slow and—at the same time—their elimination is fast, higher doses of compounds **2** and **6** are necessary to achieve the same degree of inhibition as observed with **1**. In contrast to compounds **2** and **6**, compound **8**, which is slowly cleaved in BN rat BALF + (less than 10% after 24 h), is almost inactive in ozone-treated guinea pigs as an anti-bronchoconstrictor agent (e.g., 30% inhibition at a dose of 150 μg/kg it).

These results indicate that there is a correlation between the in vitro hydrolysis rate of compounds in BALF from challenged rats and their ability to inhibit methacholine-induced bronchoconstriction in ozone-treated guinea pigs in vivo, that is compounds, which show rapid hydrolysis in vitro, are active as anti-bronchoconstrictor agents in ozone-treated animals, whereas compounds, which are not or only slowly hydrolysed in vitro, are inactive in the guinea pig ozone model. Thus, it seems that both the in vitro and the in vivo model can be useful to screen DAD.

Furthermore, the data that DAD are not cleaved in lungs from non-ozone-treated animals but are hydrolysed in guinea pigs where an inflammatory process had been evoked by pretreatment with ozone, provide evidence that the concept of disease activated drugs may work in vivo. A limitation of this in vivo model is that only compounds with rapid cleavage can be tested, since—for technical reasons—the model only allows observation of animals for 1 h.

Therapeutic window

In order to determine the therapeutic window, the effect of compound **1** and the DAD on blood pressure was compared to the anti-bronchoconstrictor effect in guinea pigs in vivo (Table 2). At 30 μg/kg it, compound **1** inhibited bronchoconstriction in ozone- and non-ozone-treated guinea pigs and increased blood pressure by about 20%. In contrast, compounds **2** and **6**, both at 100 μg/kg it caused a similar degree of inhibition of bronchoconstriction in ozone-treated guinea pigs but were devoid of effects on blood pressure, both in ozone- and non-ozone-treated animals (Table 2). This indicates an improvement of the therapeutic window for both DAD as compared to the locally administered parent compound **1.**

Hydrolysis studies in human BALF supernatants

The results obtained with some DAD in vitro and in vivo encouraged us to focus on compound **10** and carry on our studies using human material. Consequently we used human BALF supernatants from asthmatic patients and from healthy volunteers.

The hydrolysis rate and quantity of **1** formed after incubation of **10** in BALF supernatants of seven asthmatic patients was compared with those obtained in BALF supernatants of 8 healthy volunteers. Age, sex, clinical severity of asthma and pulmonary function assessed by forced expiratory volume in one second, FEV_1, characterized the patients. Moreover, BALF cell content was determined on cytospin slides (Table 3). The number of cells was similar in the asthmatic and in the control groups, 126 ± 20 and 133 ± 16, respectively (mean $\pm$ SEM $\times 10^3$/mL). Calibration curves of **1** were performed for each individual experiment and the curves were used to determine the quantity of **1** formed in the corresponding BALF supernatant. The hydrolysis of **10** in BALF supernatants from asthmatic patients yielded a small but significant amount of **1** after 24 h of incubation (14 ng, 3%) (Fig. 6). On the contrary, the hydrolysis yield in BALF supernatants from healthy volunteers after 24 h was less than 0.5%, similar to the hydrolysis observed in saline (Fig. 6). As already observed in BALF from BN rats, compound **10** was cleaved more rapidly in BALF supernatants from asthmatic patients than in BALF supernatants from non-asthmatics over a

Table 2. Therapeutic window of compounds **1**, **2**, **6** and **8** in ozone- and non-ozone-treated guinea pigs following it administration[a]

Compounds	Effect on blood pressure		Inhibition of bronchoconstriction	
	Non-ozone-treated	Ozone-treated	Non-ozone-treated	Ozone-treated
Vehicle	-1 ± 5 (4)	$+4 \pm 3$ (5)	19 ± 15 (4)	8 ± 10 (5)
1 (30 μg/kg)	$+20 \pm 7$ (3)	$+21 \pm 5$ (3)	45 ± 22 (3)	68 ± 11 (3)
2 (100 μg/kg)	$+6 \pm 2$ (3)	$+3 \pm 3$ (3)	0.3 ± 7 (3)	75 ± 8 (3)
6 (100 μg/kg)	$+6 \pm 4$ (3)	$+4 \pm 5$ (3)	21 ± 15 (3)	76 ± 7 (3)
8 (150 μg/kg)	n.d.[b]	$+4 \pm 3$ (3)	n.d.	18 ± 15 (3)

[a]Shown are effects on mean arterial blood pressure (as % change from baseline values) and inhibition of methacholine-induced bronchoconstriction (as% inhibition relative to the response before drug administration) for a selected dose of the compounds, 15 min after administration, as means $\pm$ SEM (number of experiments in brackets). For effects on blood pressure, '$-$'denotes reduction and '$+$' increase of blood pressure. The increase in blood pressure induced by compound **1** is statistically significant ($p < 0.01$; Student's t-test).
[b]n.d., no data.

period of 24 h. The quantitative difference observed between the BN rat BALF hydrolysis studies and the human BALF supernatant studies is probably due to the presence of a much larger number of inflammatory cells in the BN rat BALF as compared to the human BALF supernatants.

In connection with the preceding studies, compound **10** was also incubated in human plasma obtained from two healthy donors. Increased plasma protein extravasation is a characteristic feature of asthma.[16] The fact that **10** is rapidly hydrolysed in human plasma (donor 1: 80% after 1 h; donor 2: 40% after 1 h) indicates that, in the diseased lung, plasma proteins (possibly among other proteins) are directly involved in the hydrolysis of **10**. The rapid hydrolysis of the DAD in plasma does not necessarily argue against the concept of reduced side effects with DAD. Although the compounds are cleaved in the plasma and possibly in the blood, systemic exposure to active principles after inhalation of DAD should be much lower compared with the drug levels produced in the lung (as indicated by the improved therapeutic window for DAD in the in vivo model).

In conclusion, these data demonstrate that such DAD could offer interesting novel therapies for chronic inflammatory diseases. Reduced systemic side effects of DAD could result from the local release of active drug within the inflammatory site as well as from the potential synergy associated with the simultaneous release of two anti-inflammatory principles (e.g., PDE4 inhibitors and steroids), resulting in reduced clinical effective doses.

Table 3. Characteristics of asthmatic and control subjects and BALF cytology

Donors	Macr.[a] (%)	Eos.[a] (%)	PMN[a] (%)	Lymp.[a] (%)	Epith.[a] (%)	Squam.[a] (%)	Sex	Aas scores	FEV_1 (%)[b]	Age
1 (asthma)	74	—	12	9	5	—	M	1	100	34
2 (asthma)	16	26	50	6	2	—	F	3	40	56
3 (asthma)	80	2	13	—	5	—	F	3	65	41
4 (asthma)	48	—	28	—	21	3	F	2	70	54
5 (asthma)	96	—	—	3	—	1	F	1	109	42
6 (asthma)	38	11	1	49	1	—	F	1	97	61
7 (asthma)	50	4	3	6	17	—	M	2	78	37
1′ (control)	88	—	6	—	6	—	M		94	31
2′ (control)	93	—	1	5	1	—	M		90	25
3′ (control)	67	—	—	33	—	—	M		96	24
4′ (control)	88	—	7	1	4	—	M		100	21
5′ (control)	82	—	2	15	1	—	M		117	25
6′ (control)	51	—	1	45	3	—	M		98	30
7′ (control)	90	—	—	10	—	—	M		110	32
8′ (control)	83	—	1	16	—	—	F		102	21

[a]Macr., Macrophages; Eos., eosinophils; PMN, peripheral mononuclear cells; Lymp., lymphocytes; Epith., epithelial cells; Squam., squamous cells (other cells).
[b]Forced expiratory volume in 1 s (% of predicted values).

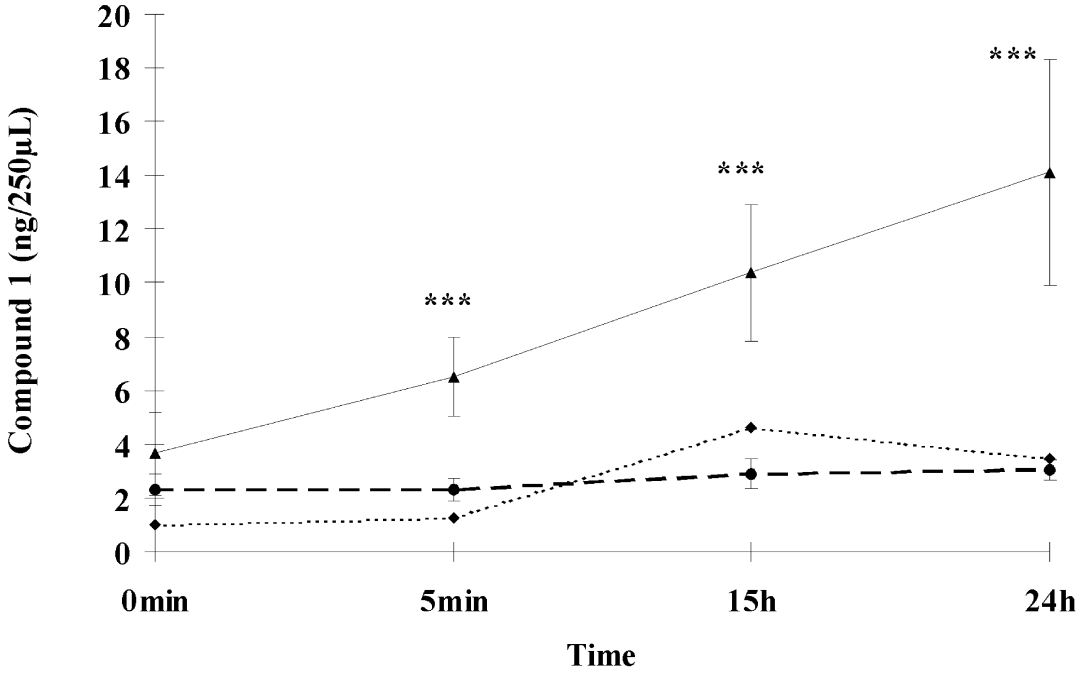


Figure 6. Comparison of the hydrolysis of compound **10** (1.25 µg/250 µL) to compound **1**, in BALF supernatants from asthmatics ($n=7$) (▲), healthy volunteers ($n=8$) (●) and in saline 0.9% ($n=2$) (◆). Shown are means ± SEM. (*** denotes significant differences, $p < 0.001$, between asthmatics and healthy volunteers).

Experimental

Chemistry

^{1}H NMR spectra were recorded with a Bruker AM-360 or a Varian Gemini-200; chemical shifts are given in ppm (δ) relative to Me_4Si as internal standard. *J* values are given in Hertz (Hz). Analyses were performed by the analytical department of Novartis, Basle and are within the ±0.4% range of the theoretical value. Analytical thin layer chromatography (TLC) was performed on silica gel 60 F_{254} glass plates (HPTLC, E. Merck). Preparative column chromatography was performed on silica gel (230–400 mesh ASTM) under pressure. Solvents were AR grade and used without further purification. *N*,*N*-dimethylformamide, triethyl amine, dichloromethane were dried over molecular sieves before use. Melting points were determined with a Büchi 512 apparatus and not corrected. Mass spectra (MS) were recorded in either the EI mode (on a VG TS 250) or the FAB mode in a matrix of thioglycerol or nitrobenzyl alcohol using a VG 70-SE mass spectrometer. All chemical yields reported are unoptimized. Extracts were dried over sodium sulphate and solvents removed under reduced pressure.

2,2-dimethyl-propionic acid 2-[1-(3,5-bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin - 6 - yloxy]-ethyl ester (2). A mixture of **1** (500 mg, 1.14 mmol) and triethyl amine (320 μL, 2.3 mmol) in dichloromethane (15 mL) was stirred under argon at room temperature. Pivaloyl chloride (215 μL, 1.75 mmol) was added to the solution and the reaction was stirred overnight at room temperature. After disappearance of **1** (TLC (ethyl acetate/hexanes 1:4) the solvent was removed and the residue purified by flash chromatography to give a white solid (460 mg, 77%). ^{1}H NMR ($CDCl_3$) δ 1.2 (s, 9H), 1.35 (d, 12H), 1.4 (t, 3H), 2.98 (q, 2H), 3.85 (s, 3H), 4.4 (m, 2H), 4.55 (m, 2H), 4.58 (m, 2H), 6.55 (t, 1H), 6.76 (d, 2H), 7.1 (s, 1H), 7.3 (s, 1H), 7.35 (s, 1H). MS *m*/*z* 524 (MH+), 396.

Addition of a solution of oxalic acid (80 mg, 0.88 mmol) in ether (3 mL) to a stirred solution of the preceding compound (460 mg, 0.88 mmol) in ether (2 mL) gave a white precipitate, **2**, which was recovered by filtration. (500 mg, 84%); mp 130 °C (decomp.), ^{1}H NMR (DMSO) δ 1.12 (s, 9H), 1.25.4 (d + t, 12H + 3H), 2.87 (q, 2H), 3.75 (s, 3H), 4.37 (m, 2H), 4.45 (m, 2H), 4.65 (m, 2H), 6.57 (t, 1H), 6.75 (d, 2H), 7.3 (s, 1H), 7.45 (s, 1H), 7.55 (s, 1H). MS *m*/*z* 523(M+), 508, 481, 129. Anal. ($C_{33}H_{43}NO_{10}$·0.5 H_2O) C: calcd 63.65; found 63.76; H: calcd 7.12; found 6.87.

2-Methyl-2-phenyl-propionic acid 2-[1-(3,5- bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl ester (3). A mixture of 2-methyl-2-phenyl propionic acid (100 mg, 0.6 mmol), oxalyl chloride (60 μL, 0.7 mmol) and few drops of DMF in dichloromethane (2 mL) was stirred at room temperature for 4 h. After removal of the solvent, a solution of the dried residue in dichloromethane (4 mL) was slowly added to a solution of 1 (220 mg, 0.5 mmol), triethyl amine (86 μL, 0.6 mmol) in dichloromethane (4 mL). (TLC (ethyl acetate/hexanes 1:4). After 24 h under stirring at room temperature, the solvent was removed and the residue purified by flash chromatography to give a white solid (125 mg, 41%). ^{1}H NMR ($CDCl_3$) δ 1.35 (d, 12H), 1.38 (t, 3H), 1.6 (s, 6H), 2.98 (q, 2H), 3.8 (s, 3H), 4.3 (m, 2H), 4.45–4.65 (m, 4H), 6.52 (t, 1H), 6.75 (d, 2H), 7 (s, 1H), 7.1.4 (m, 7H). The oxalic salt, **3**, was prepared as described for **2** (70%); mp 124–126 °C. ^{1}H NMR ($CDCl_3$) δ 1.35 (d, 12H), 1.45 (t, 3H), 1.6 (s, 6H), 3.2 (q, 2H), 3.85 (s, 3H), 4.38 (m, 2H), 4.5.6 (m, 4H), 6.65 (t, 1H), 6.74 (d, 2H), 7.1 (s, 1H), 7.12.4 (m, 6H), 7.55 (s, 1H). MS *m*/*z* 586 (MH+), 544, 396. Anal. ($C_{38}H_{45}NO_{10}$·0.5 H_2O) C: calcd 66.65; found 66.89; H: calcd 6.77 found 6.71; N: calcd 2.05; found 1.985.

1-phenyl-cyclopentanecarboxylic acid 2-[1-(3,5- bis(1 - methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl ester (4). This compound was synthesised following the procedure described for **3** using the same molar ratio: 47% of a white solid. ^{1}H NMR ($CDCl_3$) δ 1.3.45 (d + t, 15H), 1.6.8 (m, 4H), 1.7–2 (m, 2H), 2.6–2.8 (m, 2H), 2.95 (q, 2H), 3.8 (s, 3H), 4.3 (m, 2H), 4.5 (m, 2H), 4.6 (m, 2H), 6.55 (t, 1H), 6.75 (d, 2H), 7 (s, 1H), 7.1–7.4 (m, 7H). The oxalic salt **4** was prepared as already described for **2** (43%); mp 101–103 °C. ^{1}H NMR ($CDCl_3$) δ 1.35 (d, 12H), 1.45 (t, 3H), 1.65–1.8 (m, 4H), 1.85–2 (m, 2H), 2.6–2.7 (m, 2H), 3.2 (q, 2H), 3.85 (s, 3H), 4.3–4.4 (m, 2H), 4.45–4.6 (m, 4H), 6.65 (t, 1H), 6.75 (d, 2H), 7.1 (s, 1H), 7.12–7.4 (m, 6H), 7.55 (s, 1H). MS *m*/*z* 612 (MH+), 440, 396, 217. Anal. ($C_{40}H_{47}NO_{10}$·0.5 H_2O) C: calcd 67.59; found 67.33; H: calcd 6.8 found 6.72; N: calcd 1.97; found 1.89.

2,2-diphenyl-propionic acid 2-[1-(3,5-bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl ester (5). Compound **5** was synthesised following the procedure reported for **3**, using the same molar ratio: 87% of a colorless oil, ^{1}H NMR ($CDCl_3$) δ 1.38 (d, 12H), 1.4 (t, 3H), 1.95 (s, 3H), 2.97 (q, 2H), 3.8 (s, 3H), 4.3 (m, 2H), 4.5–4.65 (m, 4H), 6.55 (t, 1H), 6.75 (d, 2H), 7 (s, 1H), 7.15–7.25 (m, 11H), 7.35 (s, 1H). MS *m*/*z* 648 (MH+), 440, 396, 181. Anal. ($C_{41}H_{45}NO_6$.0.5 H_2O) C: calcd 74.97; found 74.6; H: calcd 7.06 found 7.1; N: calcd 2.13; found 2.

Carbonic acid 2-[1-(3,5-bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl ester ethyl-ester (6). Compound **6** was synthesised according to the method already described for **2** using the same molar ratio: 30% of a white solid, ^{1}H NMR ($CDCl_3$) δ 1.2–1.45 (d + 2t, 12H + 6H), 2.95 (q, 2H), 3.8 (s, 3H), 4.25 (q, 2H), 4.4 (m, 2H), 4.5–4.8 (m, 4H), 6.52 (t, 1H), 6.75 (d, 2H), 7.32 (s, 1H), 7.35 (s, 1H), 7.5 (s, 1H). Anal. ($C_{29}H_{37}NO_7$) C: calcd 68.08; found 67.94; H: calcd 7.29 found 7.29; N: calcd 2.74; found 2.66. The oxalic salt was prepared as already described for **2** (78%); mp 93–98 °C. ^{1}H NMR ($CDCl_3$) δ 1.3–1.4 (d + t, 12H + 3H), 1.45 (t, 3H), 3.2 (q, 2H), 3.85 (s, 3H), 4.25 (q, 2H), 4.47 (m, 2H), 4.57 (m, 2H), 4.65 (m, 2H), 6.65 (t, 1H), 6.75 (d, 2H), 7.2 (s, 1H), 7.35 (s, 1H), 7.55 (s, 1H). MS *m*/*z* 512 (MH+), 440, 396.

Carbonic acid bis-{2-[1-(3,5-bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl} ester (8).

A mixture of **1** (1.3 g, 3 mmol), 1,1′-carbonyldiimidazole (251 mg, 1.5 mmol) and Na_2CO_3 (1.24 g, 9 mmol) in 2-butanone (15 mL) was heated at 65 °C overnight. The suspension was filtrated through Celite and the Celite washed several times with acetone. After solvent evaporation, the residue was dissolved in ethyl acetate, washed with water and dried. Chromatography (eluent: ethyl acetate/hexanes 1:1) yielded **8** as a white solid (1.06 g, 78%); mp 79–82 °C; ^{1}H NMR ($CDCl_3$) δ 1.35 (d, 12H), 1.4 (t, 3H), 2.95 (q, 2H), 3.84 (s, 3H), 4.4 (m, 2H), 4.55 (m, 2H), 4.65 (m, 2H), 6.5 (t, 1H), 6.75 (d, 2H), 7.05 (s, 1H), 7.3 (s, 1H), 7.35 (s, 1H). MS *m*/*z* 905 (MH+), 891, 863, 440. Anal. ($C_{53}H_{64}N_2O_{11}$·0.5 H_2O) C: calcd 69.56; found 69.5; H: calcd 7.27; found 7.26 N: calcd 3.06; found 2.91.

Carbonic acid 2-[1-(3,5-bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl ester 2-(9-fluoro-β,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[a]phenantren-17-yl)-2-oxo ethyl ester (10). To a solution of 1 (336 mg, 0.765 mmol), 4-dimethylaminopyridine (385 mg, 3.1 mmol) in methylene chloride (6 mL) was added 1,1′-carbonyldiimidazole (128 mg, 0.765 mmol). The reaction was stirred at room temperature for 5 h. The disappearance of **1** and formation of the stable intermediate **7** was monitored by TLC (ethyl acetate/hexanes 2:1). [**7**, ^{1}H NMR ($CDCl_3$) δ 1.35–1.4 (t + d, 3H + 12H), 2.85 (q, 2H), 3.7 (s, 3H), 4.5–4.7 (m, 4H), 4.8 (m, 2H), 6.55 (t, 1H), 6.75 (d, 2H), 7 (d, 1H), 7.33 (s, 1H), 7.43 (s, 1H), 7.46 (s, 1H), 7.5 (d, 1H), 8.1 (d, 1H). MS *m*/*z* 533 (M+), 518, 491]. Dexamethasone (300 mg, 0.765 mmol) and 6 mL of dichloromethane were added to the solution and the suspension stirred at 40 °C for 48 h. TLC showed the formation of two less polar compounds. After evaporation of the solvent, the residue was dissolved in ethyl acetate, neutralised to pH 7, washed with water and brine, dried and chromatographied (solvent: ethyl acetate/hexanes 1:1 to 2:1): 55% of **10** as a white solid; mp 135 °C (decomp); ^{1}H NMR ($CDCl_3$) δ 0.9 (d, *J* = 7, 3H), 1.05 (s, 3H), 1.1–1.9 (m, 5H), 1.38 (d, *J* = 7, 12H), 1.4 (t, 3H), 1.55 (s, 3H), 2.2 (m, 2H), 2.21 (s, 1H exchangeable), 2.25.5 (m, 3H), 2.6 (ddd, *J* = 6, 10.8, 11, 1H), 2.97 (q, 2H), 3.1 (m, 1H), 3.85 (s, 3H), 4.23 (m, 1H), 4.45 (m, 2H), 4.5.7 (m, 4H), 4.72 and 4.97 (AX, *J* = 17, 2H), 6.1 (s, 1H), 6.34 (dd, *J* = 2, 9, 1H), 6.55 (t, 1H), 6.8 (d, 2H), 7.1 (s, 1H), 7.13 (d, *J* = 9, 1H), 7.33 (s, 1H), 7.38 (s, 1H). MS *m*/*z* 858 (MH+), 484, 440, 396. Anal. ($C_{49}H_{60}FNO_{11}$·1 H_2O) C: calcd 67.18; found 67.08; H: calcd 7.13; found 7.30. The carbonyl homodimer of **1**, **8** (20%), less polar than **10**, was also isolated as side product.

Thiocarbonic acid 2-[1-(3,5-bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl ester 2-(9-fluoro-11β,17-dihydroxy-10,13,16-trimethyl-3-oxo-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[a]-phenantren-17-yl)-2-oxo ethyl ester (11). Compound **11** was synthesised following the procedure reported for **10** using 1,1′-thiocarbonyldiimidazole and the same molar ratio: 32% of a white solid (210 mg); mp 150 °C (decomp); ^{1}H NMR ($CDCl_3$) δ 0.93 (d, *J* = 7, 3H), 1.05 (s, 3H), 1.2–1.8 (m, 4H), 1.38 (d, *J* = 7, 12H), 1.4 (t, 3H), 1.55 (s, 3H), 1.6–1.85 (m, 2H), 2.15 (m, 1H), 2.2–2.5 (m, 4H), 2.6 (ddd, *J* = 6, 15, 15, 1H), 2.95 (q, 2H), 3.1 (m, 1H), 3.85 (s, 3H), 4.23 (m, 1H), 4.45–4.7 (m + q, 4H), 4.9 (m, 1H), 5 (m, 1H), 5.1 and 5.5 (AX, *J* = 18, 2H), 6.1 (s, 1H), 6.34 (dd, *J* = 2, 7, 1H), 6.55 (t, 1H), 6.78 (d, 2H), 7.1 (s, 1H), 7.15 (d, *J* = 9, 1H), 7.35 (s, 1H), 7.38 (s, 1H). MS *m*/*z* 874 (MH+), 500, 440, 396. Anal. ($C_{49}H_{60}FNO_{10}S$·0.5 H_2O) C: calcd 66.65; found 66.61; H: calcd 6.96; found 6.98.

Thiocarbonic acid bis-{2-[1-(3,5-bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl} ester (9). The thiocarbonyl homodimer of **1,** less polar than **11,** was isolated as side product (28%) and characterised: mp 78–82 °C, ^{1}H NMR ($CDCl_3$) δ 1.35 (d, 12H), 1.4 (t, 3H), 2.95 (q, 2H), 3.8 (s, 3H), 4.45 (m, 2H), 4.55 (m, 2H), 4.95 (m, 2H), 6.5 (t, 1H), 6.75 (d, 2H), 7.05 (s, 1H), 7.3 (s, 1H), 7.35 (s, 1H). MS *m*/*z* 921 (MH+), 817, 440, 396. Anal. ($C_{53}H_{64}N_2O_{10}S$·1 H_2O) C: calcd 67.84; found 68.06; H: calcd 7.089; found 7.26 N: calcd 2.99; found 2.73.

Carbonic acid 2-[1-(3,5-bis(1-methylethoxy)-phenyl)-3-ethyl-7-methoxy-isoquinolin-6-yloxy]-ethyl ester 2-(5-hydroxy-4a,6a-dimethyl-2-oxo-8-propyl-2,4a,4b,5,6,6a,9a,10,10a,10b,11,12-dodecahydro-7,9-dioxa-pentaleno[2,1-a]-phenanthren-6b-yl)-2-oxo-ethyl ester (12). Compound **12** was synthesised following the procedure reported for **10,** using budesonide in a third of the dexamethasone molar ratio: 72% of a white solid (150 mg); mp 120 °C (decomp); ^{1}H NMR (DMSO) δ 0.85 (s + t, 3 + 3H), 0.8–2.4 (m, 13H), 1.3 (d + t, 12 + 3H), 1.4 (s, 3H), 2.6 (m, 1H), 2.85 (q, 2H), 3.8 (s, 3H), 4.3 (m, 1H), 4.4 (m, 2H), 4.55 (m, 2H), 4.6–4.73 (m, 4H), 4.83 (d, 1H exchangeable), 4.71.87 (AX, 2 d, 1H) and 5.2 (AX, 2 d + 2 t, 2H), 5.9 (s, 1H), 6.15 (d, *J* = 9, 1H), 6.55 (t, 1H), 6.72 (d, 2H), 7.31(d, *J* = 9, 1H), 7.33 (s, 1H), 7.4 (s, 1H), 7.47 (s, 1H). MS *m*/*z* 896 (MH+), 580, 484, 440. Anal. ($C_{52}H_{65}NO_{12}$·0.25 H_2O) C: calcd 69.35; found 69.24; H: calcd 7.33; found 7.35.

Biological methods

Phosphodiesterase enzymatic assays. PDE3 enzyme preparation (from human platelets). Platelet concentrate was obtained from the local blood transfusion center. Platelets were washed once with phosphate buffered saline (PBS; NaCl 0.14 M, KCl 2.7 mM, KH_2PO_4 1.5 mM, Na_2HPO_4 8.1 mM, adjusted to pH 7.4 at room temperature) suspended in 10 mL of buffer H [sucrose 0.25 M, ethylenediamine-tetraacetic acid (EDTA) 1 mM, tris 40 mM adjusted to pH 7.4 with HCl], dithiothreitol 1 mM and the following protease inhibitor solutions: 5 μL/mL of phenylmethyl-sulphonylfluoride (7 mg/mL in 2-propanol), 1 μL/mL leupeptin and pepstatin A (1 mg/mL each, in ethanol). After sonication (15 s at 4 °C, Branson probe sonicator), homogenates were centrifuged at 2200 *g*. The pellet was resuspended in the same volume of buffer H and the sonication repeated. Pooled supernates were stored at −20 °C until use as PDE enzyme source

PDE4 enzyme preparation (from human neutrophils). Neutrophils were isolated from human blood using spontaneous sedimentation in 1% dextran and isopycinic centrifugation over Histopaque 1119 and 1077. After washing, a suspension of 3×10^7 cells/mL PBS was sonicated 4 times 15 s at 4 °C (Branson probe sonicator). Immediately after sonication, protease inhibitor solutions (5 μL/mL of phenylmethyl-sulphonylfluoride (7 mg/mL in 2-propanol), 1 μL/mL leupeptin and pepstatin A (1 mg/mL each, in ethanol), dithiothreitol (0.15 mg/mL) and Na_2EDTA (0.37 mg/mL) were added. The centrifugation supernatant (1500*g*, 10 min, 4 °C) was made 25% (v/v) in ethylene glycol and 1 mg/mL CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). This preparation was stored at −20 °C until use as PDE enzyme source. Since human PDE4B is 92% homologous to rat PDE4B,[9] the rat enzyme was substituted for the human PDE4B in order to improve the availability of the enzyme.

PDE4B cloning and expression. PDE4 cDNA coding for the isoenzyme rat PDE4B[17] was integrated (PDE4B; single copy) at the pep4 locus of a *Saccharomyces cerevisiae* strain lacking both of the wild-type yeast PDE genes.[18] The yeast strain was grown in 1 L cultures at 30 °C, pelleted and frozen until homogenization. Pelleted yeast (5 mL) was suspended in 50 mL of buffer (10 mM Tris–hydroxymethyl-aminomethane, 1 mM EDTA, 1 mg/mL each of leupeptin and pepstatin A, 175 mg/mL phenylmethyl-sulphonyl fluoride, 1 mM dithiothreitol, pH 7.4 with HCl). After centrifugation, 15 g of glass beads (425–600 mm, acid washed, Sigma Chemical Co.) washed with buffer were added to the pellet. To this slurry, 1 mL of buffer and 60 mg of cholamidopropane sulphonic acid were added and the slurry was vigorously agitated during 4 h at 4 °C. Disintegration of the yeast cells was observed microscopically (phase-contrast optics) as dark cells and was >30% (usually 50%). The slurry was transferred to a coarse glass funnel and the homogenate collected by suction and washing of the glass beads with a total of 15 mL buffer. Cell fragments were separated from cytosol by centrifugation (2000 *g*, 10 min, 4 °C). The pellet was resuspended in 15 mL of buffer and assayed for PDE activity together with the cytosol.

Assay of PDE activity. Activity was assayed either by the method of Thompson[19] or as published by Colicelli.[17]

DAD hydrolysis in BN rat BALF

BALF. Male Brown-Norway (BN) rats (approximately 200 g) were used for the study of cell accumulation in the lungs. Ovalbumin (OA: 10 μg/0.5 mL) was mixed with aluminium oxide (10 mg/0.5 mL) and injected (sc) simultaneously with Bortadella pertussis vaccine (0.25 mL/animal ip). Injection of OA, together with adjuvant, was repeated 15 and 21 days later. On day 28, the sensitized animals were restrained in plastic tubes and exposed (60 min) to an aerosol of OA (3.2 mg/mL) using a nose only exposure. Animals were killed 48 h later with pentobarbital (250 mg/kg ip). The lungs were lavaged using three aliquots (4 mL) of Hank's solution [HBSS×10 (Hank's balanced salt solution 10× concentrate), 100 mL; EDTA 100 mM, 100 mL; HEPES (4-(2-hydroxyethyl)piperazine-1-ethylsulfonic acid) 1 M, 10 mL; H_2O, 1 l]. The lavages from sensitized challenged animals (BALF+) were pooled and used immediately or stored at −20 °C for at least 2 months without loss of activity. BALF from sensitized non challenged animals (BALF−) was obtained in the same way from sensitized animals, which were treated on day 28 with saline instead of OA.

Eosinophils in BALF (mean±SE). The number of eosinophils was $25.18\pm4.97\times10^6$ in BALF+ and $0.17\pm0.054\times10^6$ cells /mL in BALF−.

DAD solutions. Each compound (approximately 10 mg, exactly weighed) and a reference compound **13** (approximately 20 mg, exactly weighed) were dissolved in 10 mL DMSO for the hydrolysis studies.

Hydrolysis studies. Five microlitres of a solution of DAD + **13** in DMSO was added to 500 μL of BALF or Hank's solution, as control, and the samples were incubated at 37 °C for 1, 2, 4 and 24 h. Thereafter, 250 μL of a mixture of isopropanol and *tert*-butyl methyl ether (1/4) was added to the sample. The suspension was mixed for 2 min using a vortex-genie (Bender & Holbein AG) speed control 7. The sample was then centrifuged at 4000 rpm for 2 min and the organic phase was removed. The procedure was repeated three times. The dried combined organic layers were dissolved in 500 μL acetonitrile. Each set of experiments was run twice. Extraction of 250 μL of BALF± without compound was also used as control.

HPLC analysis. Twenty microlitres of the 500 μL acetonitrile sample were injected onto the HPLC system [LiChrosorb-250-RP-select-B (5 μm, 250×4 mm, E. Merck) fitted with a C-8 precolumn]. Isocratic elution was achieved with 30% water containing 10-camphorsulfonic acid (0.5 g/L) and 70% acetonitrile. The flow was typically 1.5 mL/min, the temperature of the column 40 °C, and the UV detection at 254 nm. Under these conditions, the internal standard **13**, **1**, and the study compounds had retention times of 4, 5.3 and <20 min, respectively, except for **8** and **9** (the two homodimers of **1**) which had 23 and 48 min, respectively, as retention time. Peaks corresponding to each compound were integrated and the ratio between area of **1** and area of **1**+ area of DAD was used for quantitation. The extraction yield of the compounds was controlled by comparison of the compound **13** peak area in the samples to the peak area produced by 5 μL of the mother solution in 500 μL acetonitrile. The formation of dexamethasone was investigated by retention time comparison with a commercial sample, addition of a small amount of commercial dexamethasone to the aliquot and confirmation that, under less polar conditions of elution, no additional peak was formed.

In vivo model

Measurement of lung function and mean arterial blood pressure in guinea pigs. Lung function and blood pressure

were measured as described previously.[20] The animals were anaesthetized, paralysed and ventilated via a tracheal cannula. Air flow was measured at the trachea by a pneumotachograph connected to a differential pressure transducer. Pressure changes within the thorax were monitored via an intrathoracic cannula, connected to a differential pressure transducer to determine the pressure difference between the trachea and thorax (transpulmonary pressure). From measurements of air flow and transpulmonary pressure, airway resistance [R_L, cm H_2O/(L/s)] and dynamic compliance (C_{dyn}, mL/cm H_2O) were calculated after each respiratory cycle with a digital electronic respiratory analyzer. Mean arterial blood pressure was continuously recorded from the carotid artery using a pressure transducer.

Anti-bronchoconstrictor effects in ozone-treated guinea pig. Conscious male Dunkin–Hartley guinea pigs were treated with ozone (3 ppm, 30 min). After a recovery phase of 60 min, the animals were prepared for lung function measurement as described above. Transient bronchoconstriction was induced twice in 10 minute intervals by iv injections of methacholine (3.2 μg/kg). The increase in R_L obtained by the second injection was taken as 100% response. The test compound was administered intratracheally (0.1 mL) following the second injection of methacholine. 5, 15, 30 and 60 min thereafter, bronchoconstriction was induced by single iv injections of methacholine. Anti-bronchoconstrictor activity was expressed as % inhibition of the bronchoconstriction to methacholine relative to the response before administration of the test compound.

Anti-bronchoconstrictor effects in healthy guinea pigs. The anti-bronchoconstrictor effect of the test compounds was also assessed in normoreactive guinea pigs. In this case, the animals were treated with air instead of ozone and a higher dose of methacholine (10 μg/kg iv) was administered in order to obtain the same degree of bronchoconstriction as in ozone-treated animals. Otherwise, the same procedure, as described above, was used.

Data evaluation. Data from individual experiments were presented as means ± SEM and unpaired Student's *t*-test was used for comparison between treatments at individual time points. Significance was assumed at the 5% probability level.

Test compound and route of administration. For intratracheal administration, the test compounds were suspended in a medium of tragacanth gum 0.5% in distilled water. This was sonicated for 30–60 s at 100 W before administration in order to reduce the particle size. The solutions and suspensions were prepared freshly on the day of the experiment.

Hydrolysis of 10 in human BALF supernatants

Subjects (Table 3). The hydrolysis studies were performed in BALF supernatants of seven patients with asthma (aged 34–61 years; mean ± SD, 41 ± 14 years). Asthma was defined according to the criteria of the American Thoracic Society [21,22] and all the patients had either a reversible airway obstruction characterised by an increase of 15% of FEV_1 after inhalation of 200 μg of salbutamol or a positive challenge with carbachol.[23] None of the subjects had smoked within the previous 2 years. No subject had any bronchial or respiratory tract infection during the month preceding the test and had never taken systemic or inhaled corticosteroids of any form. The BALF supernatants of eight healthy volunteers (aged 21–32 years; mean ± SD, 26 ± 4 years) were used as a control group. Their pulmonary function was within the normal range. None of the subjects were current or previous smokers. No subject had any bronchial or respiratory tract infection during the month preceding the test. The study was done after informed consent and approval from the ethic committee of the University of Montpellier.

Fiberoptic bronchoscopy and bronchoalveolar lavage (BAL). Fiberoptic bronchoscopy was performed as previously described.[23] Briefly, after premedication with atopine and diazepam, and local anaesthesia with lidocaine, 2% applied to the upper respiratory tract, a BFTR fiberoptic bronchoscope (Olympus, Tokyo, Japan) was inserted into the trachea. The BAL was performed identically in all patients in one of the subsegmental bronchi of the middle lobe with the injection of several aliquots of sterile saline (total volume of 150 mL) reaspirated by gentle syringe suction. Each bronchoscopy was done with oxygen, and adrenaline was readily available. A nebulization with 1 mg of salbutamol was performed after the procedure. Immediately after lavage, cytocentrifuge slides were made with an aliquot of the lavage, and the remaining BALF was centrifuged at +4 °C for 400 *g* for 5 min. The supernatant was stored at −20 °C until further utilisation.

Total cell number in BALF. The total cell number was $126 \pm 20 \times 10^3$ cells/mL in BALF from asthmatic patients and $133 \pm 16 \times 10^3$ cells/mL in BALF from the control group (means ± SEM).

Asthma scores. The severity of asthma was rated according to the clinical score of Aas[24] used to grade chronic asthma from very mild form (score of 1) to incapacitating disease requiring severe medication (score 5; Table 3).

Pulmonary function. Pulmonary function was assessed by measuring% FEV_1 and was expressed as % of predicted values as described.[25]

Hydrolysis studies of 10. A solution of **10** [12.5 μL (1.25μg] in DMSO (100 μg/mL) was added to 250 μL of human BALF supernatant and incubated at 37 °C for 0 min, 5 min, 4 h, 15 h, 24 h, 48 h. After addition of 250μL isopropanol and *tert*-butyl methyl ether (1:4), the sample was agitated 2 min in a vortex-genie (Bender & Holbein AG) speed control 7–8. The sample was then centrifuged at 4000 rpm for 2 min and the organic phase was removed. The procedure was repeated three times. The organic layers were combined and dried under argon flow at 37 °C. Each set of experiments was run twice and a set of experiments in saline 0.9% was run as

control. Extraction of 250 μL of each BALF without compound was also used as control. It was checked that no residual peak after extraction of the different supernatant samples interfered in the UV detection of **1** and that incubation of **10** in saline 0.9% yielded only a negligible amount of **1**.

Calibration curve of 1. Several concentrations of **1** (0, 10, 25 and 50 ng) were spiked in 250 μL of BALF supernatant of each individual, extracted and dried as described above.

HPLC-MS analysis. Samples were dissolved in 100 μL of acetonitrile/water (1:1) containing 200 ng/mL of the internal standard terbinafine (MW 291). Twenty microlitres were injected onto the HPLC system. The separation was achieved using a Nucleosil C18-AB column (2.1×50 mm) packed with C-18 reverse phase and fitted with a C-18 precolumn (Optimize Technologies). Flow was typically 300 μL/min and temperature of the column was kept at 60 °C. Eluent A was water containing 0.05% trifluoroacetic acid. Eluent B was acetonitrile containing 0.05% trifluoroacetic acid. Percentage of B was kept at 10% for 1 min then a gradient was run from 10 to 95% of B in 9 min. Under these conditions **1**, terbinafine and **10** had retention times of 6.7, 7.0 and 8.3 min, respectively. Flow was split 1:4 prior to ionisation allowing a flow of approximately 75 μL/min into the electrospray (ESI) source. Molecules were ionised by ESI. Heated capillary temperature was kept at 220 °C. Under these conditions, **10**, **1** and terbinafine mainly yielded the MH^+ ion (*m*/*z* 859, 440 and 292, respectively). Selected Ion Monitoring was used as a scan/detection method (span 0.3 amu, 1.5 s/scan). Multiplier was set at 2000 volts. Peaks corresponding to each compound were integrated and ratio between **1** and terbinafine was used for quantitation.

Statistical evaluation. The *F*-test was used for determination of the type of data distribution within the various groups. Comparison between different groups was performed using Student's test (one-tailed); *p* values <0.05 were taken as significant.

Acknowledgements

We wish to express our gratitude to J. Brun, I. Donze, M. Stefani, A. Dunbar for technical assistance and Dr. R. Hersperger for helpful and insightful discussions.

References and Notes

1. (a) Torphy, T. J.; Undem, B. J. *Thorax* **1991**, *46*, 512. (b) Raeburn, D.; Souness, J. E.; Tomkinson, A.; Karlsson, J.-A. *Prog. Drug Res.* **1993**, *40*, 9. (c) Christensen, S. B.; Torphy, T. *Annu. Rep. Med. Chem.* **1994**, *29*, 185.
2. Palfreyman, M. N.; Souness, J. E. Phosphodiesterase Type IV Inhibitors. In *Progress in Medicinal Chemistry*; Ellis, G. P., Luscombe, D. K., Eds.; Elsevier Science: Amsterdam, 1996; pp 1–52.
3. Wright, L. C.; Seybold, J.; Robichaud, A.; Adcock, I. M.; Barnes, P. J. *Am. J. Physiol.* **1998**, *275*, 694.
4. Naef, R. DE 4438737, **1995**.
5. Stache, U.; Fritsch, W.; Alpermann, H. G. DE 27 35 110, 1979.
6. (a) Pozo, M.; Pulido, R.; Gotor, V. *Tetrahedron* **1992**, *48*, 6477. (b) Moris, F.; Gotor, V. *Tetrahedron* **1992**, *48*, 9869. (c) Pozo, M.; Gotor, V. *Tetrahedron* **1993**, *49*, 10725.
7. Teranishi, K.; Kayakiri, T.; Mizutani, M.; Hisamatsu, M. *Biosci. Biotech. Biochem.* **1994**, *58*, 1537.
8. Engels, P.; Lübbert, H.; Fichtel, K. *FEBS Lett.* **1994**, *350*, 291.
9. Obernolte, R.; Bhakta, S.; Alvarez, R.; Bach, C.; Zuppan, P.; Milkins, M.; Jarnagin, K.; Shelton, E. R. *Gene* **1993**, *129*, 239.
10. Tominaga, T.; Watanabe, A.; Tsuji, J.; Haruki, H.; Koda, A. *Gen. Pharmacol.* **1995**, *26*, 353.
11. Ethanol, 2-[1-[3-(1-methylethoxy)-5-hydroxy-phenyl)-3-isopropyl-7-methoxy-3,4-dihydro-6-isoquinolinyl] oxy]. ^{1}H NMR (DMSO) δ 1 (d, 3H), 1.05 (d, 3H), 1.2–1.3 (2d, 6H), 1.9 (m, 1H), 2.4 (AX, $J=18$ and 12 Hz, 1H), 2.64 (AX, $J=18$ and 6 Hz, 1H), 3 (m, 1H), 3.65 (s, 3H), 3.75 (m, 2H), 4.05 (m, 2H), 4.55 (m, 1H), 4.9 (t, 1H exchangeable), 6.4 (t, 1H), 6.5 (d, 1H), 6.58 (d, 1H), 6.8 (s, 1H), 7 (s, 1H). MS *m*/*z* 414 (MH+). Anal. ($C_{24}H_{31}NO_5$) C: calcd 69.71 found 69.68; H: calcd 7.56; found 7.60; N: calcd 3.39; found 3.36.
12. Seltzer, J.; Bigby, B. G.; Stulbarg, M.; Holtzman, M. J.; Nadel, J. A.; Ueki, I. F.; Leikauf, G. D.; Goetzl, E. J.; Boushey, H. A. *J. Appl. Phys.* **1986**, *60*, 1321.
13. Evans, T. W.; Brokaw, J. J.; Chung, K. F.; Nadel, J. A.; McDonald, D. M. *Am. Rev. Respir. Dis.* **1988**, *138*, 140.
14. Yeadon, M.; Wilkinson, D.; Darley Usmar, V.; O'Leary, V. J.; Payne, A. N. *Pulm. Pharmacol.* **1992**, *5*, 39.
15. (a) Heaslip, R. J.; Lombardo, L. J.; Golankiewicz, J. M.; Ilsemann, B. A.; Evans, D. Y.; Sickels, B. D.; Mudrick, J. K.; Bagli, J.; Weichman, B. M. *J. Pharmacol. Exp. Ther.* **1994**, *268*, 888. (b) Hughes, B.; Howat, D.; Lisle, H.; Holbrook, M.; James, T.; Gozzard, N.; Blease, K.; Hughes, P.; Kingaby, R.; Warrellow, G. *Br. J. Pharmacol.* **1996**, *118*, 1183. (c) Raeburn, D.; Underwood, S. L.; Lewis, S. A.; Woodman, V. R.; Battram, C. H.; Tomkinson, A.; Sharma, S.; Jordan, R.; Souness, J. E.; Webber, S. E. *Br. J. Pharmacol.* **1994**, *113*, 1423. (d) Turner, C. R.; Cohan, V. L.; Cheng, J. B.; Showell, H. J.; Pazoles, C. J.; Watson, J. W. *J. Pharmacol. Exp. Ther.* **1996**, *278*, 1349.
16. Glynn, A. A.; Michaels, L. *Thorax* **1960**, *15*, 142.
17. Colicelli, J.; Birchmeier, C.; Michael, T.; O'Neill, K.; Riggs, M.; Wigler, M. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 3599.
18. Sullivan, M.; Egerton, M.; Shakur, Y.; Marquardsen, A.; Houslay, M. D. *Cell Signal* **1994**, *6*, 793.
19. Thompson, W. J.; Terasaki, W. J.; Epstein, P. M.; Strada, S. J. *Adv. Sec. Mess. Phosphoprot. Res.* **1979**, *10*, 69.
20. Buchheit, K. H.; Hofmann, A. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1996**, *354*, 355.
21. American Thoracic Society. Committee on Diagnostic Standards for Non-tuberculous Respiratory Diseases. *Am. Rev. Respir. Dis.* **1962**, *85*, 762.
22. Chai, H.; Farr, R. S.; Froelich, L. A.; Mathison, D. A.; McLean, J. A.; Rosenthal, R. R.; Sheffer, A. L.; Spector, S. L.; Townley, R. G. *J. Allergy Clin. Immunol.* **1975**, *56*, 323.
23. Godard, P.; Aubas, P.; Calvayrac, P.; Taib, J.; Michel, F. B. *Nouv. Presse Med.* **1981**, *10*, 3141.
24. Aas, K. Heterogeneity of bronchial asthma: sub-populations—or different stages of the disease *Allergy* **1981**, *36*, 3.
25. Van Vyve, T.; Chanez, P.; Bousquet, J.; Lacoste, J. Y.; Michel, F.-B.; Godard, P. *Am. Rev. Respir. Dis.* **1992**, *146*, 116.