British Journal of Pharmacology (1996) 118, 1183–1191 © 1996 Stockton Press All rights reserved 0007–1188/96 $12.00

The inhibition of antigen-induced eosinophilia and bronchoconstriction by CDP840, a novel stereo-selective inhibitor of phosphodiesterase type 4

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1 The novel tri-aryl ethane CDP840, is a potent and selective inhibitor of cyclic AMP phosphodiesterase type 4 (PDE 4) extracted from tissues or recombinant PDE 4 isoforms expressed in yeast (IC_{50}s: 4–45 nM). CDP840 is stereo-selective since its S enantiomer (CT 1731) is 10–50 times less active against all forms of PDE 4 tested while both enantiomers are inactive (IC_{50}s: >100 μM) against PDE types 1, 2, 3 and 5.

2 Oral administration of CDP840 caused a dose-dependent reduction of interleukin-5 (IL-5)-induced pleural eosinophilia in rats (ED_{50} = 0.03 mg kg^{-1}). The eosinophils in pleural exudates from CDP840-treated animals contained higher levels of eosinophil peroxidase (EPO) than cells from control animals, suggesting a stabilizing effect on eosinophil degranulation. CDP840 was approximately equi-active with the steroid dexamethasone in this model and was 10–100 times more potent than the known PDE 4-selective inhibitors rolipram and RP73401. The activity of CDP840 was not influenced by adrenalectomy, β-sympathomimetics or β-sympatholytics.

3 Antigen-induced pulmonary eosinophilia in sensitized guinea-pigs was reduced dose-dependently by CDP840 (0.01–1 mg kg^{-1}, i.p.) and intracellular EPO levels were significantly higher. CDP840 was more potent in these activities than CT1731 or rolipram and comparable in potency to RP73401.

4 Rolipram or CDP840 were less active than dexamethasone in preventing neutrophil accumulation, or exudate formation in carrageenan-induced pleurisy in rats and thus do not exhibit general anti-inflammatory activity.

5 In sensitized guinea-pigs, aerosols of the antigen ovalbumin caused a dose-dependent bronchoconstriction demonstrated by an increase in pulmonary inflation pressure. Administration of CDP840 (0.001–1.0 mg kg^{-1}, i.p.), 1 h before antigen challenge, resulted in dose-dependent reduction in response to antigen. This activity was not due to bronchodilatation since higher doses of CDP840 (3 mg kg^{-1}) did not significantly change the bronchoconstrictor response to histamine. Rolipram was approximately 10 times less active than CDP840 in preventing antigen-induced bronchoconstriction.

6 These results confirm the observations that selective PDE 4 inhibitors reduce antigen-induced bronchoconstriction and pulmonary eosinophilic inflammation. CDP840 is more potent than rolipram in inhibiting native or recombinant PDE 4. Unlike the recently described potent PDE 4 inhibitor RP73401, CDP840 is more active than rolipram in the rat IL-5 model following oral administration. The novel series of tri-aryl ethanes, of which CDP840 is the lead compound, could be the basis of an orally active prophylactic treatment for human asthma.

Keywords: CDP840; phosphodiesterase inhibitor; allergic airway disease; eosinophilia; anti-inflammatory; bronchoconstriction; anti-asthmatic; esoinophil stabilization; RP73401; rolipram

Introduction

There is considerable evidence that elevation of intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) reduces the responsiveness of tissues involved in allergic airway disease. In bronchial smooth muscle and inflammatory leukocytes cyclic AMP is hydrolysed by predominantly phosphodiesterase type 4 (PDE 4) and it has been proposed that selective inhibitors of this enzyme may have a use in the treatment of human asthma (Torphy & Undem, 1991). The PDE 4 subtype was originally characterized by the selective inhibitor rolipram (Schwabe *et al*., 1976; Beavo & Reifsnyder, 1990; Figure 1) which is a relatively weak inhibitor of the isolated enzyme ($IC_{50} \approx 1$ μM). Recently, the benzamide derivative, RP73401, (Figure 1) has been shown to be approximately 1000 times more potent than rolipram in inhibiting PDE 4 (Souness *et al*., 1995). However, RP73401 is only equipotent with rolipram in reducing allergic bronchoconstriction and inflammation in rodents (Raeburn *et al*., 1994). This activity has been demonstrated following local administration of RP73401 by intratracheal instillation. We now describe a novel tri-aryl ethane (CDP840; Figure 1) which is a potent, stereoselective and orally active inhibitor of native PDE 4 and various human recombinant isoforms of PDE 4. We have compared the effects of CDP840 with rolipram and RP73401 in interleukin-5 (IL-5) or antigen-induced responses *in vivo*. Some of these results have been communicated to the British Pharmacological Society (Hughes *et al*., 1995).

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Methods

Isolation of phosphodiesterase isoenzymes

Phosphodiesterase type 1 (PDE 1; Ca^{2+}/calmodulin-dependent) and PDE 2 (guanosine 3′:5′-cyclic monophosphate (cyclic

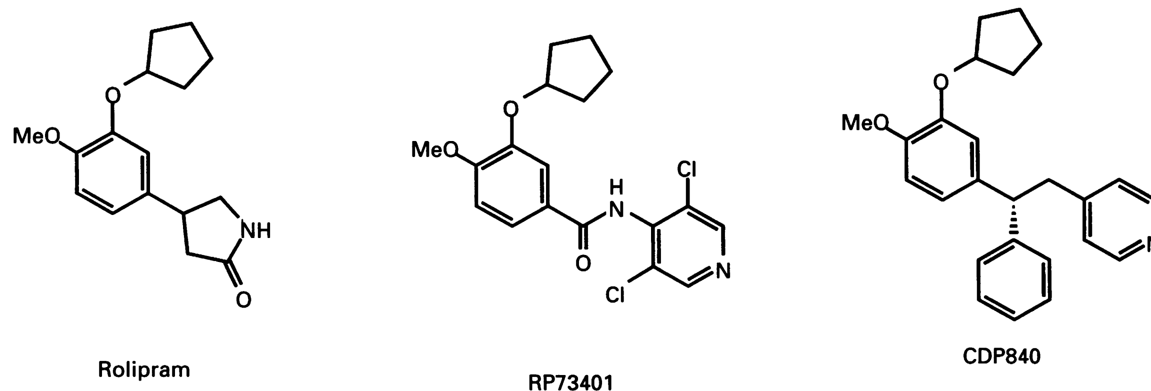

Figure 1 Chemical structures of rolipram, RP73401 and CDP840.

GMP) stimulated) were extracted from rabbit brain, PDE 3 (cyclic GMP inhibited) from rabbit heart and PDE 5 (cyclic GMP specific) from guinea-pig lung. PDE 4 was extracted from the human granulocyte cell line HL60. All isoenzymes were partially purified from 100,000 *g* supernatants by h.p.l.c. chromatography on Mono Q (Pharmacia, Milton Keynes, U.K.) with appropriate NaCl gradients in 50 mM TES-NaOH pH 7.6. The following protease inhibitors were maintained throughout: AEBSF (0.2 mM), aprotinin (1 μg ml^{-1}), benzamidine (1 mM), leupeptin (10 μg ml^{-1}), pepstatin (10 μg ml^{-1}), E-64 (1 μg ml^{-1}) and EDTA 5 mM (all from Boehringer Mannheim GmbH, Mannheim, Germany).

Isolation and expression of PDE 4 cDNAs

The cDNA cloning and expression of the four human PDE 4 isoforms (A, B, C and D) will be described in detail elsewhere. Briefly, full length cDNAs encoding PDE 4B and D were isolated by the polymerase chain reaction from a human eosinophil cDNA library using published sequence data (McLaughlin *et al.*, 1993; Bolger *et al.*, 1993). A human PDE 4A cDNA was obtained from a human frontal cortex cDNA library (Clontech) by hybridization with a partial cDNA isolated from the human monocytic cell line U937 (Livi *et al.*, 1990).

A combination of reverse transcription coupled to PCR and 5′ RACE methods (Edwards *et al.*, 1991) was used to isolate a PDE 4C cDNA from the human glioblastoma cell line U87 (Engels *et al.*, 1994).

All four isoform cDNAs were inserted into the vector pYES (In Vitrogen) for production in *Saccharomyces cerevisiae*. Transformed yeast cells were grown in a 1.8L fed-batch fermenter and expression of enzymes induced by addition of galactose. Cells were harvested approximately 48 h later.

Purification of rPDE 4 isoenzymes

All recombinant enzymes were purified by use of a similar strategy to that used for PDEs extracted from tissues. The buffer employed throughout was 50 mM TES-NaOH pH 7.6. The recombinant enzyme was extracted from the soluble yeast cell lysate (100,000 *g*, 30 min) onto Q-sepharose (Pharmacia, Milton Keynes, U.K.) and recovered by application of a linear NaCl gradient (0 to 1 M). The fractions containing the recombinant enzyme were pooled and applied to a column of Blue-Sepharose (Pharmacia, Milton Keynes, U.K.). After extensive washing with 1 M and 2 M NaCl the recombinant enzyme was eluted by use of a linear gradient of CHAPS (0 to 1%). Finally, the enzyme was subjected to h.p.l.c. chromatography on Mono-Q (Pharmacia, Milton Keynes, U.K.) in the presence of a linear NaCl gradient (0 to 1 μM). To maintain the integrity of the enzyme it was necessary to include the protease inhibitors throughout the purification procedure (see above).

Measurement of PDE activity

PDE activity was determined as described previously (Smith *et al.*, 1993). Briefly, the reaction mixture contained 50 mM TES-NaOH pH 7.6, 10 mM $MgCl_2$, and 0.1 μM [^{3}H]-cyclic AMP. The inhibitors were dissolved in dimethylsulphoxide (DMSO) and added to the reaction mixture such that the concentration of DMSO did not exceed 1% (v/v). Reactions were initiated by addition of enzyme and terminated after incubation at 30°C for 10–30 min by addition of [^{14}C]-AMP in trifluoroacetic acid to final concentrations of 1.0 μM and 0.5% (v/v) respectively. Kinetic studies were performed by maintaining a constant concentration of [^{3}H]-cyclic AMP while varying the amount of unlabelled cyclic AMP added. In all cases substrate consumption was less than 20%. The IC_{50} values (concentration required to inhibit substrate hydrolysis by 50%) for the compounds were determined from at least three concentration-response curves for each compound.

[^{3}H]-rolipram binding

R(−)-rolipram was iodinated and dispatched to Amersham International where it was tritiated by catalytic reduction with palladium and charcoal to a specific activity of 851GBq mmol^{-1} (23 Ci mmol^{-1}).

The ability of compounds to inhibit the binding of [^{3}H]-**R**(−)-rolipram to guinea-pig brain membranes was investigated by use of the method of Schneider *et al.* (1986). The concentration of [^{3}H]-**R**(−)-rolipram employed was 5 nM and sufficient membranes were used to bind 10% of the total label.

Interleukin-5-induced pleurisy

Interleukin-5 was used to induce a pleural eosinophilia in female Wistar rats of approximatemly 200–220 g (Lisle *et al.*, 1993). The animals were allowed free access to food and water at all times. Animals were lightly anaesthetized with N_2O/O_2 halothane and injected intrapleurally with 25 u of murine recombinant interleukin-5 (mrIL-5; Genzyme) in 0.1 ml of pyrogen-free saline containing 0.1% BSA. The animals were killed by anaesthetic overdose 24 h after the challenge and the contents of the pleural cavity lavaged with 1 ml citrated saline (3.15%). Total white cell counts were determined by Coulter-counting and eosinophil numbers estimated from Kimura-stained haemocytometer preparations. The cell pellet was obtained by centrifugation and frozen for storage prior to cellular eosinophil peroxidase (EPO) assay (see below).

Groups of six animals were dosed orally (1.0 ml) intravenously (0.5 ml) or intraperitoneally (0.5 ml) with vehicle, CDP840 (0.003–3 mg kg^{-1}), CT1731 (0.003–3 mg kg^{-1}),

dexamethasone (0.0003 – 1.0 mg kg^{-1}), indomethacin (1 – 10 mg kg^{-1}), rolipram (0.1 – 1.0 mg kg^{-1}), RP73401 (0.3 – 10 mg kg^{-1}), or aminophylline (10 – 30 mg kg^{-1}) just before the intrapleural injection of IL-5 and again 6 h later by the same route. Drugs were dissolved in water (p.o.) or saline (i.v. and i.p.) or the appropriate vehicle.

In some experiments the animals were dosed p.o. with CDP840 (0.3 mg kg^{-1} twice daily) or vehicle for 7 days before induction of IL-5 pleurisy. Animals with no pretreatment but given CDP840 (0.3 mg kg^{-1}) just before IL-5 challenge were used for comparison.

In order to investigate the effects of β-adrenoceptor agents on the anti-inflammatory effects of CDP840, animals were treated with propranolol (0.3 mg kg^{-1}, i.v.) or salbutamol (0.1 mg kg^{-1}, i.p.) at time 0 and 6 h after IL-5 injection, with and without CDP840 (0.3 mg kg^{-1} p.o.).

The effect of endogenous steroids on the model was studied in adrenalectomized rats (from Harlan Olac). These animals were the same strain, sex and weight as those used above, but were given 0.9% saline with 10 g l^{-1} sucrose in tap water in place of normal drinking water. The effects of CDP840 (0.3 mg kg^{-1}) on IL-5 induced pleurisy in adrenalectomized and sham-operated animals were compared with control groups.

Carrageenan pleurisy

Female Wistar rats of approximately 200 – 220 g were used for the carrageenan pleurisy model. The animals were anaesthetized with N_2O/O_2 halothane and injected intrapleurally with 0.1 ml of a 0.5% carrageenan solution in saline. Six hours after the carrageenan challenge, the animals were killed by anaesthetic overdose and the pleural cavity lavaged with 1.0 ml of citrated saline. The volume of fluid recovered was recorded and the volume of exudate in the cavity determined. Total white cell counts were determined by Coulter-counting. Groups of six animals were dosed orally with vehicle, CDP840 (0.01 – 10 mg kg^{-1}), rolipram (0.03 – 10 mg kg^{-1}), dexamethasone (0.01 – 3 mg kg^{-1}) or indomethacin (0.03 – 10 mg kg^{-1}) just before carrageenan challenge. Results are expressed as a percentage of the response in the vehicle control group.

Eosinophil peroxidase assay

Eosinophil peroxidase (EPO) was measured by the method of Strath *et al.* (1985). Briefly, pleural or broncho-alveolar lavage fluids were centrifuged (1000 *g*, 10 min, 4°C) to obtain a cell pellet which was re-suspended in 1 ml of Tris buffer (pH 8.0) and the cells disrupted by sonication. The EPO activity was then assayed by taking 100 μl of a 1 : 100 dilution of the cell lysate and adding 100 μl substrate solution (50 mM Tris pH 8.0, 0.1% Triton X-100, 1 mM H_2O_2 and 0.1 mM O-phenylenediamine dihydrochloride) in a microtitre well plate. The reaction was allowed to proceed for 30 min at room temperature and terminated by the addition of 50 μl 4 M H_2SO_4. The absorption was then read at 492 nm in a plate reader. A standard curve for enzyme activity was constructed with horse radish peroxidase (1 – 100 ng ml^{-1}) and EPO values were calculated as horse radish peroxidase equivalents. Enzyme activity was expressed per 10^6 eosinophils and values from groups of drug-related animals were compared with appropriate controls.

Antigen-induced inflammatory cell accumulation

To determine the effects of drugs on antigen-induced pulmonary inflammation, groups of guinea-pigs (Dunkin-Hartley male, 350 – 400 g) were sensitized to ovalbumin (OA), over a six week period by the method described by Sanjar *et al.* (1990). On day 0, animals received cyclophosphamide (100 mg kg^{-1}, i.p.) followed on days 1, 14 and 27 by 1 ml (i.p.) of a suspension containing OA (10 μg), Al $(OH)_3$ (10 mg) and *Bordetella pertussis* (1×10^9 organisms). Animals were used between days 42 and 50 following sensitization.

On day 42, animals were administered pyrilamine (10 mg kg^{-1}, i.p.) to protect against anaphylaxis and 30 min later they were challenged by inhalation of either aerosolized saline (sham) or OA (0.1%) for a 30 min period in a perspex chamber ($0.5 \times 0.3 \times 0.3$ m). The ovalbumin aerosol was generated from a 1 mg ml^{-1} solution in saline by a nebulizer (De Vilbiss Pulmosonic) and carried through the chamber with room air at a rate of 10 l min^{-1}. Forty-eight hours after antigen-challenge, animals were killed by overdose of anaesthetic and blood ($\sim$10 μl) taken for different cell count. This time point was selected because it has been previously shown to be maximal for eosinophil infiltration of the airways (Sanjar *et al*, 1990). The trachea was cannulated and the lungs lavaged with Hanks buffered salt solution containing citrated saline (5×2 ml aliquots). The broncho-alveolar lavage (BAL) fluid aliquots were pooled and the total volume measured. The fluid was centrifuged (1000 *g* at 4°C for 10 min) and the cell pellet resuspended in Hanks solution. Samples of blood and resuspended BAL pellets were taken for total and differential cell counts.

Groups of 6 – 10 animals were treated i.p. with CDP840, rolipram or RP73401 2 h before antigen challenge, 6 h later and again 24 h before lavage. The effects of drugs on inflammatory cell numbers in BAL fluids were expressed as a percentage of values obtained from similar groups of control animals. Eosinophil peroxidase levels were determined in BAL eosinophils by the method described above.

Spasmogen-induced bronchospasm in vivo

Male Dunkin-Hartley guinea-pigs (400 – 600 g) were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.). The trachea was cannulated to allow for mechanical ventilation (Harvard small animal ventilator) at 56 breaths min^{-1} with a tidal volume of 10 ml kg^{-1}. Pulmonary inflation pressure (PIP) was measured with a pressure transducer (Viggo-Spectromed, P10EZ) connected to a side arm of the tracheal cannula. Animals were instrumented for the measurement of blood pressure from the left carotid artery and for intravenous drug or anaesthetic administration via the jugular vein. Blood pressure was measured with a pressure transducer and heart rate was derived from this signal. All parameters were recorded on a Gould chart recorder (3800).

Histamine (5 – 640 μg ml^{-1}) or saline was delivered by inhalation via a nebulizer (De Vilbiss) for a 10 s period. Saline was always administered before the construction of a histamine dose-response curve performed with doubling doses. The lungs of each animal were hyper-inflated by occluding expired air flow for three breaths between each dose. PIP was allowed to return to basal levels before administration of the next dose. An exclusion criteria for the study was failure of PIP to return to its basal level.

Dose-response curves to histamine were determined in five untreated animals and values were expressed as percentage increases in PIP. The mean concentration of histamine required to elicit a 200% increase in PIP was also determined (PC_{200}). The effect of CDP840 and salbutamol on histamine-induced bronchoconstriction was determined following i.v. or i.p. administration alone or in combination 1 h before histamine.

Antigen-induced bronchospasm in vivo

Sensitized guinea-pigs were anaesthetized with sodium pentobarbitone (40 mg kg^{-1}, i.p.) and the animals instrumented for blood pressure, PIP and intravenous administration as described above. Ovalbumin (10 μg – 3 mg ml^{-1}) was nebulized in a De Vilbiss nebulizer and delivered over a 10 s period with increasing doses being administered every 15 min. Antigen was administered to separate groups of animals at low doses (10 – 100 μg ml^{-1}) or high doses (0.1 – 3 mg ml^{-1}). Concentrations of antigen required to increase PIP by 20% (PC_{20}) or 100%

(PC_{100}) were calculated by interpolation from dose-response curves. The effects of CDP840 or rolipram administered i.p. 1 h prior to low or high dose antigen challenge were compared.

Drugs and solutions

Materials used were ovalbumin, histamine, carbachol, salbutamol, pyrilamine, indomethacin, dexamethasone, aminophylline, O-phenylenediamine dihydrochloride (Sigma, U.K.); aluminium hydroxide (BDH); *Bordetella pertussis* vaccine (Wellcome, U.K.); sodium pentabarbitone (Sagatal; Rhône Merieux); Hanks balanced salt solution (Gibco, U.K.). CDP840 (**R**-(+)-4-[2-(3-cyclopentyloxy-4-methoxyphenyl)-2-phenylethyl] pyridine), CT1731 (its S-(−)-enantiomer), RP73401 and (±)-rolipram were synthesized in the Department of Medicinal Chemistry, Celltech Therapeutics Ltd. CDP840 was dissolved in sterile water (acidified to pH 6). Rolipram was dissolved in 30% ethanol: 70% saline to a concentration of 1 mg ml^{-1}. RP73401 was dissolved in dimethylformamide (DMF).

Data analysis and statistics

Data are expressed as means ± s.e.mean for *n* observations. Statistical significance of differences between means of groups was determined by applying parametric (ANOVA and Student's *t* test) or non-parametric statistics as appropriate. $P<0.05$ was accepted as a level of significance.

Results

Inhibition of isolated PDE 4

CDP840 and its S enantiomer CT1731 produced a concentration-dependent, competitive inhibition of the hydrolysis of cyclic AMP by native PDE 4 but showed little or no activity against native PDEs 1, 2, 3 or 5 (Table 1). CDP840 was approximately 20 times more active than CT1731.

Human recombinant PDE 4 isoforms A, B, C and D were expressed in yeast and active enzyme was recovered from broken cell preparations. Rolipram was approximately equiactive against PDE 4 A, B and D and significantly less active against PDE 4C (Table 2). CDP840 was 15–75 times more potent than rolipram and again showed weakest activity against PDE 4C. The S enantiomer of CDP840 (CT1731) was about 10–50 times less active than CDP840 and comparable in potency to rolipram. The most active inhibitor against all PDE 4 isoforms was RP73401 (Table 2).

The PDE 4 inhibitors each competed with [^{3}H]-rolipram at a high affinity binding site (Sr) on guinea-pig brain membranes. RP73401 and rolipram were more active than CDP840 which in turn was more active than its enantiomer (Table 3).

Table 1 Inhibition of native phosphodiesterases

Isoenzyme	CDP840	IC$_{50}$ (nM) CT1731	RP73401	(±)-Rolipram
PDE 1	$>10^5$	$>10^5$	$>10^5$	$>10^5$
PDE 2	$>10^5$	$>10^5$	$>10^5$	$>10^5$
PDE 3	$>10^5$	$>10^5$	$>10^5$	$>10^5$
PDE 4	12 ± 2.3	225 ± 20.8	NT	250 ± 18.9
PDE 5	$>10^5$	$>10^5$	NT	$>10^5$

All isoenzymes were assayed at 0.1 μM substrate which was cyclic AMP for phosphodiesterases (PDEs) 1, 2, 3 and 4 and cyclic GMP for PDE 5. PDE1 assays included 2 mM $CaCl_2$ and 100 u ml^{-1} calmodulin whilst PDE 2 was assayed in the presence of 5 mM cyclic GMP. Each value is expressed as the mean ± s.e.mean of 3 separate experiments. NT = not tested.

IL-5 induced pleurisy

Thoracic cavity lavage fluids from normal, untreated rats contained $0.52 \pm 0.1 \times 10^6$ ml^{-1} neutrophils (mean ± s.e.mean, $n=6$), $4.7 \pm 0.6 \times 10^6$ ml^{-1} mononuclear cells and $0.26 \pm 0.08 \times 10^6$ ml^{-1} eosinophils. Following the intrapleural injection of mrIL-5 there was a time-dependent recruitment of inflammatory cells. There was an early neutrophil infiltration which was maximal at 6 h ($7.08 \pm 0.48 \times 10^6$ ml^{-1}) and returned to normal by 48 h. Mononuclear cell infiltration peaked at 48 h ($7.37 \pm 0.37 \times 10^6$ ml^{-1}) but the increase above basal levels was not statistically significant. The peak eosinophil infiltration was at 24 h ($1.54 \pm 0.18 \times 10^6$ ml^{-1}) and remained elevated until 48 h.

Oral administration of CDP840 caused a dose-dependent reduction of eosinophil numbers in pleural exudates 24 h after IL-5 injection ($ED_{50} = 0.03$ mg kg^{-1}; Figure 2). The maximum reduction in eosinophil numbers was around 60% which represents approximately 90% of the recruited cells. CDP840 was comparable in potency to dexamethasone in reducing

Table 2 Inhibition of human recombinant phosphodiesterase (PDE) 4 isoforms

Isoform	CDP840	IC$_{50}$ (nM) CT1731	RP73401	(±)-Rolipram
PDE 4A	4 ± 1.1	148 ± 21.2	0.2 ± 0.07	300 ± 58.2
PDE 4B	9 ± 1.9	212 ± 25.2	0.8 ± 0.2	201 ± 29.1
PDE 4C	45 ± 1.0	626 ± 21.9	2 ± 0.6	1519 ± 101
PDE 4D	9 ± 2.1	385 ± 21.2	0.2 ± 0.07	135 ± 12

Isoforms were expressed in yeast. Each value is expressed as the mean ± s.e.mean of 3 separate experiments.

Table 3 Inhibition of [^{3}H]-rolipram binding to guinea-pig brain membranes

	CDP840	CT1731	RP73401	(±)-Rolipram
IC$_{50}$ (nM)	60 ± 13.1	415 ± 19.6	2 ± 0.4	10 ± 1.1

Each value is expressed as the mean ± s.e.mean of 3 separate experiments.

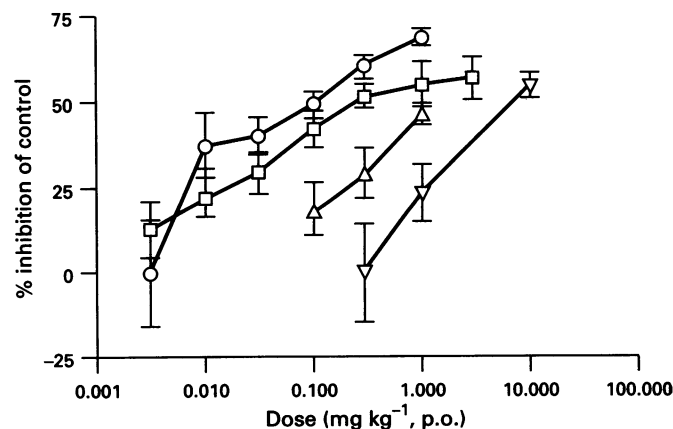

Figure 2 The effects of orally administered dexamethasone (○), CDP840 (□) rolipram (△) or RP73401 (▽) on eosinophil numbers in rat pleural exudates 24 h after the injection of interleukin-5. Each point represents the mean of 5–40 values expressed as a percentage of controls. The vertical lines represent s.e.mean.

Table 4 The effects of drugs on interleukin-5 (IL-5)-induced pleural eosinophilia in rats and on intracellular levels of eosinophil peroxidase (EPO)

Compound	*Dose ×2/24 h* (mg kg^{-1})	*% inhibition eosinophils*	*EPO activity/10^6 eosinophils (% control)*
CDP840	0.3 p.o.	55*	172*
CDP840	0.3 i.p.	48*	NT
CT1731	0.3 p.o.	36	154*
RP73401	1.0 p.o.	24	118
RP73401	1.0 i.p.	50*	133
RP73401	0.3 i.p.	33	NT
Rolipram	1.0 p.o.	48*	171*
Dexamethasone	0.3 p.o.	67*	142
Indomethacin	1.0 p.o.	26	120
Aminophylline	10 p.o.	0	140

Each value is the mean from at least 6 animals and is expressed as a percentage of control values in the same experiment; *indicates that $P<0.05$ by analysis of variance. NT = not tested.

eosinophil numbers and these drugs were approximately 10 and 100 times more active than rolipram and RP73401 respectively (Figure 2). When RP73401 was administered intraperitoneally (0.3 – 1.0 mg kg^{-1}) it was approximately 10 times more potent than by the oral route but was still less active than CDP840 (0.3 mg kg^{-1}, i.p.) (Table 4, Figure 2).

Eosinophils collected from animals treated with CDP840, rolipram or CT1731 contained significantly higher levels of EPO activity than cells from untreated controls (Table 4). Dexamethasone, indomethacin, aminophylline or RP73401 did not significantly affect EPO at the doses tested (Table 4). Indomethacin or aminophylline also had no effect on eosinophil numbers in this model.

The effects of CDP840 in this model were not changed significantly following 7 days repeated dosing before IL-5 challenge. Similarly, the activity of CDP840 was not affected by the sex of the animal, adrenalectomy or concomitant treatment with β-adrenoceptor agonists or antagonists (data not shown).

Carrageenan pleurisy

Intrapleural injection of carrageenan in control animals produced an exudate of 1.0 – 1.5 ml containing 50 – 70 × 10^6 leukocytes which were predominantly neutrophils at 6 h (>95%). Dexamethasone (0.01 – 0.3 mg kg^{-1}, p.o.) caused a marked, dose-dependent inhibition of exudate formation (ED_{50} = 0.03 mg kg^{-1}) and neutrophil migration (ED_{50} = 0.06 mg kg^{-1}). Indomethacin (0.03 – 10 mg kg^{-1}, p.o.) was less potent than dexamethasone (ED_{50}s, 3 – 10 mg kg^{-1}) and only high doses of rolipram significantly reduced neutrophil numbers (15 – 30% inhibition at 10 mg kg^{-1}). CDP840 (0.1 – 10 mg kg^{-1}, p.o.) had little or no effect on neutrophil migration or exudate formation (<15% inhibition at 10 mg kg^{-1}).

Antigen-induced pulmonary inflammation

Aerosolized antigen but not saline caused a marked inflammatory cell influx into guinea-pig airways 48 h after challenge and the greatest percentage increase was in the numbers of eosinophils. Neutrophil, macrophage and lymphocyte numbers were also elevated. CDP840 (0.01 – 1.0 mg kg^{-1}, i.p.) caused a dose-dependent reduction in the numbers of eosinophils, macrophages and lymphocytes but had no significant effect on neutrophils (Figure 3). In separate experiments RP73401 (0.01 – 0.1 mg kg^{-1}, i.p.) caused a comparable effect to CDP840 but rolipram (0.5 – 1.0 mg kg^{-1},

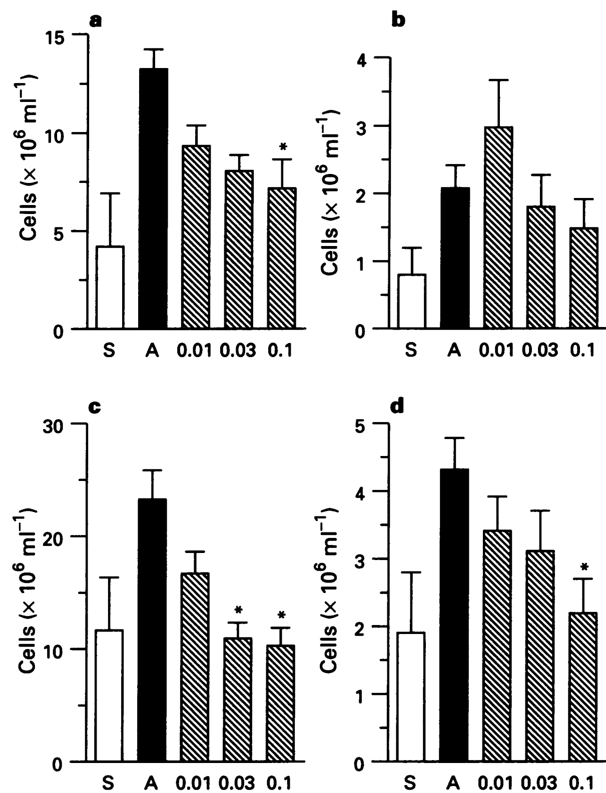

Figure 3 The effects of CDP840 on antigen-induced recruitment of (a) esosinophils, (b) neutrophils, (c) macrophage/monocytes and (d) lymphocytes collected in broncho-alveolar lavages from sensitized guinea-pigs. The open columns show the responses to aerosolized saline (S) and the solid columns show the responses to aerosolized antigen (A). The hatched columns are values from antigen challenged animals treated with CDP840 (0.01 – 0.1 mg kg^{-1}, i.p. × 3/48 h; see methods for dosing schedule). Each value is the mean from 10 animals and vertical lines represent s.e.mean. *Indicates that $P<0.05$ compared to antigen alone values (A) by analysis of variance.

Table 5 The effect of drugs on antigen-induced eosinophil recruitment into guinea-pig broncho-alveolar lavages

Compound	*Dose ×3/48 h* (mg kg^{-1}, i.p.)	*% inhibition eosinophils*
CDP840	0.01	29
	0.03	40
	0.1	46*
RP73401	0.01	35
	0.1	59*
Rolipram	0.5	25
	1.0	58*
CT1731	1.0	29

Each value is the mean from 7 – 10 animals and is expressed as a percentage of control values obtained in the same experiment. *Indicates that $P<0.05$ by analysis of variance.

i.p.) was approximately ten times less active than CDP840, while CT1731 (1.0 mg kg^{-1}, i.p.) was inactive at this dose (Table 5). In a number of experiments rolipram also caused a significant reduction in neutrophil numbers. The inhibitory effects of CDP840 did not reflect any changes in peripheral blood leukocyte counts.

Eosinophils collected from sensitized guinea-pigs challenged only with saline contained double the EPO activity measured in cells from antigen-challenged animals (Table 6). This indicates that the antigen causes activation and degranulation of eosinophils. The reduction of intracellular EPO was prevented

Table 6 The effect of drugs on intracellular eosinophil peroxidase (EPO) in eosinophils collected in broncho-alveolar lavage fluids from antigen-challenged guinea-pigs

Treatment	*Dose ×3/48 h* (mg kg^{-1}, i.p.)	Challenge	*EPO µg/10^6 eosinophils*
Vehicle	–	Saline	2.6±0.2*
Vehicle	–	Antigen	1.3±0.3
CDP840	1.0	Antigen	2.4±0.4*
CT1731	1.0	Antigen	1.1±0.3
Rolipram	1.0	Antigen	2.2±0.4*

EPO values are means of 7–10 values±s.e.mean, expressed as µg equivalents of horse radish peroxidase activity. *Indicates that $P<0.05$ compared to vehicle/antigen values by analysis of variance.

by CDP840 and rolipram but not CT1731 (Table 6). In a separate experiment, lower doses of CDP840 (0.1 mg kg^{-1}, i.p.) or RP73401 (0.1 mg kg^{-1}, i.p.) caused a similar significant reduction in EPO loss, whereas neither compound had a significant effect at 0.01 mg kg^{-1}, i.p.

Spasmogen-induced bronchoconstriction in vivo

Intravenous administration of CDP840 (3 mg kg^{-1}) 15 min before histamine challenge did not cause any significant change in the degree of bronchoconstriction or the PC_{200} values. CDP840 did not cause any significant alteration in basal haemodynamic parameters by either route of administration. Salbutamol (1 mg kg^{-1}, i.p.) caused a parallel right shift of the histamine concentration-response curve and a significant increase in the PC_{200} value. Co-administration of CDP840 and salbutamol did not augment the effects of salbutamol alone. There was no significant alteration in basal haemodynamics in any group compared to vehicle control groups.

Antigen-induced bronchospasm

Aerosolized antigen resulted in a dose-related bronchoconstriction with a maximal increase in mean PIP of 456±48%. CDP840 (1 mg kg^{-1}) caused a right shift of the curve and a change in the shape of the curve (Figure 4) with a significant increase in the PC_{100}. CDP840 (0.1 mg kg^{-1}) caused a smaller and parallel right shift of the curve and though the PC_{100} was increased this did not attain statistical significance. Since CDP840 caused a change in the curve shape when lower concentrations of ovalbumin were administered, this was explored in more detail by use of low dose antigen challenge. Administration of CDP840 at doses of 0.001–0.01 mg kg^{-1} caused a dose-related parallel shift in the curve (Figure 5) and 0.01 mg kg^{-1} elicited a significant increase in the PC_{20}. Rolipram was ten fold less potent than CDP840 in inhibiting antigen-induced bronchospasm (Figure 4, Table 7). Neither agent affected basal PIP nor was there any effect of CDP840, rolipram or vehicle on basal haemodynamic parameters.

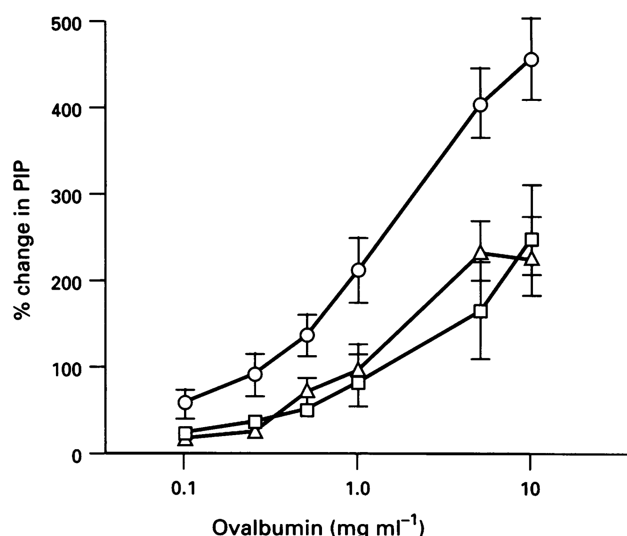

Figure 4 The effects of CDP840 or rolipram on antigen (ovalbumin)-induced bronchoconstriction in anaesthetized guinea-pigs. Response curves are shown following treatment with vehicle (○), CDP840, 1.0 mg kg^{-1}, i.p. (□) or rolipram, 10 mg kg^{-1}, i.p. (△). Each point is the mean of 6 values and vertical lines represent s.e.mean.

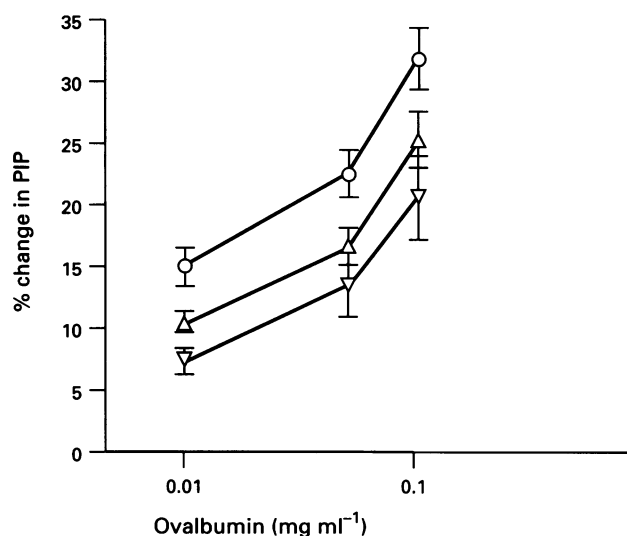

Figure 5 The effects of CDP840 on low-dose antigen-induced bronchoconstriction. Responses are shown following treatment with vehicle (○), CDP840 0.001 mg kg^{-1}, i.p. (△) or CDP840 0.01 mg kg^{-1}, i.p. (▽). Each point is the mean of 6 values and vertical lines represent s.e.mean.

Table 7 The effects of CDP840 and rolipram on bronchoconstriction (PC_{20} and PC_{100}) following low and high dose antigen challenge

Treatment (mg kg^{-1}, i.p.)	PC_{20} (low dose antigen)	PC_{100} (high dose antigen)
Vehicle	1.55±0.12	2.60±0.17
CDP840, 0.001	1.85±0.08	
CDP840, 0.01	1.95±0.10*	
CDP840, 0.1		2.93±1.76
CDP840, 1.0		3.36±0.18*
Rolipram, 1.0		2.68±0.35
Rolipram, 10.0		3.14±0.17*

Each value is the mean±s.e.mean of 6–10 separate experiments and *indicates $P<0.05$ compared to control values (ANOVA). PC_{20} and PC_{100} are the $\log_{10}$ of the concentrations of antigen required to cause an increase in pulmonary inflation pressure by 20% or 100%, respectively.

Discussion

The results presented in this paper and in the following two papers (Holbrook *et al.*, 1996; Gozzard *et al.*, 1996) confirm and extend the observations that selective inhibitors of PDE 4 inhibit inflammatory airway responses (Torphy & Undem, 1991; Giembycz & Dent, 1992; Raeburn *et al.*, 1994). It is likely that these activities are a consequence of elevation of intracellular cyclic AMP in a number of different tissues including resident and recruited cells in the lungs.

The discovery of rolipram (Schwabe *et al.*, 1976) helped to characterize the PDE 4 sub-group (Beavo & Refsneider, 1990),

but inhibition of the cell-free enzyme by rolipram is relatively modest. During the last 15 years a large number of rolipram analogues have been produced with similar properties to rolipram but recently, a benzamide derivative, RP73401 (Figure 1) has been synthesized which has a marked increase in potency compared to rolipram (Raeburn *et al.*, 1994). We now describe a novel tri-aryl ethane, CDP840, which is also more potent than rolipram *in vitro* but has important differences from rolipram and RP73401 in its pharmacology.

Both RP73401 and CDP840 differ from rolipram in being relatively less active at the high affinity binding site (Sr) than at the catalytic site (Sc) and CDP840 is less potent at the Sr site than RP73401. Also, CDP840 is stereo-selective since its S enantiomer CT1731 is less active in preventing the hydrolysis of cyclic AMP. This enantiomeric selectivity is useful in defining specific mechanisms in cell-based or *in vivo* experiments.

The IL-5-induced pleural eosinophilia in rats is a simple model of cell recruitment *in vivo*. IL-5 released from allergen-specific T-lymphocytes in the lungs of asthmatics is thought to be a local chemotactic mechanism for the recruitment of eosinophils (Corrigan & Kay, 1990). The elevation of intracellular cyclic AMP in eosinophils by selective PDE 4 inhibitors has been associated with suppression of eosinophil function *in vitro* (Souness *et al.*, 1995) and inhibition of eosinophil recruitment *in vivo* (Underwood *et al.*, 1993). The observations described here that PDE 4 inhibitors suppress the IL-5-induced recruitment and activation of eosinophils *in vivo*, support the theory that elevation of eosinophil cyclic AMP renders these cells less responsive. It is also possible that the PDE 4 inhibitors elevate cyclic AMP in other tissues involved in the eosinophil response to IL-5. For example, PDE 4 inhibition may influence the induction and expression of cytokines such as TNF and IL-1 (Molnar-Kimber *et al.*, 1992) or adhesion molecules which are required for eosinophil activation (Pober *et al.*, 1993).

Rolipram is 15–75 times less active than CDP840 in inhibiting isolated forms of PDE 4 and in the *in vivo* models described in this paper, rolipram is approximately 10 times less active than CDP840. The relative lack of potency of rolipram against some PDE 4 preparations may be due to isoform selectivity or an artifact of the cell-free system. It is known, for example, that rolipram is relatively more active in elevating cyclic AMP and inhibiting superoxide production in eosinophils than it is against the cell-free enzyme (Souness *et al.*, 1991). Furthermore, solubilization of particulate eosinophil PDE 4 or treatment of eosinophil membranes with vanadate/glutathione results in a ten fold increase in the potency of rolipram, indicating that the conformation of PDE 4 determines its susceptibility to inhibitors (Souness *et al.*, 1995).

Interestingly, RP73401 which is more potent than both rolipram and CDP840 in inhibiting isolated PDE 4 is the least active in the rat IL-5 model after oral administration. This is partly explained by poor oral bioavailability of the compound since when RP73401 is administered intraperitoneally its potency is improved. In the rat, CDP840 is still more potent than RP73401 when given intraperitoneally whereas in the guinea-pig the two compounds are comparable in potency. However, intraperitoneal administration of RP73401 to guinea-pigs failed to block ozone-induced bronchial hyperresponsiveness; a response which is prevented by both CDP840 and rolipram (Holbrook *et al.*, 1996). These anomalies may reflect differences in the distribution of the compounds between the tissues and the blood in the different species. Also, the observations indicate that PDE 4 isoform distribution and enzyme conformation *in situ* may be important determinants of inhibitor activity.

In common with previous findings, we have shown that selective PDE 4 inhibitors reduce antigen-induced pulmonary eosinophilia in sensitized guinea-pigs (Underwood *et al.*, 1993; Raeburn *et al.*, 1994). CDP840 and RP73401 gave comparable effects following intra-peritoneal administration and both were approximately 10 times more potent than rolipram. In this model there was also evidence that the PDE 4 inhibitors increased eosinophil stabilization as intracellular levels of EPO in cells collected from treated animals were significantly higher than the control values (Table 6).

Antigen challenge in guinea-pigs produced increases in other inflammatory cells as well as eosinophils. CDP840 significantly reduced the increases in macrophage and lymphocyte numbers but had no consistent effects on neutrophils. Rolipram, however, did produce a significant inhibition of neutrophil recruitment in both the antigen-challenged guinea-pigs and in the carrageenan-induced pleurisy in rats, but at higher doses.

The anti-inflammatory steroid dexamethasone, is a potent inhibitor of the recruitment and activation of all inflammatory leukocytes and although CDP840 is equi-active with dexamethasone in reducing IL-5-induced eosinophilia it is much less active than dexamethasone in suppressing neutrophil dominated responses such as carrageenan-induced pleurisy. This suggests that CDP840 will be most active as an anti-inflammatory agent in responses in which eosinophils play a key role such as inflammatory asthma.

In both models of eosinophilia described in this paper, the S enantiomer of CDP840 (CT1731) was less active than CDP840 itself. This was most evident in the antigen-challenged guinea-pigs where the effects of CT1731 (1 mg kg^{-1}) on eosinophil recruitment and activation were not significant (Tables 5 and 6), whereas CDP840 produced significant effects at 0.1 mg kg^{-1}. In the IL-5 eosinophilia model in rats, CT1731 (0.3 mg kg^{-1}, p.o.) produced less of an effect than CDP840 at the same dose (Table 4). However, CT1731 did cause a dose-dependent inhibition of eosinophil recruitment in this model (data not shown). In a model of airway responsiveness in the guinea-pig, CT1731 is markedly less potent that CDP840 (Holbrook *et al.*, 1996). The demonstration of stereo-selective effects *in vivo* for CDP840 and CT1731, which correlate with the activity of the enantiomers against recombinant PDE 4 *in vitro*, support the interpretation that the *in vivo* effects are due to selective inhibition of the enzyme.

Pretreatment of guinea-pigs with CDP840 or rolipram, before antigen-challenge through the airways, reduced the acute bronchoconstriction. Local administration of rolipram or RP73401 has been shown to inhibit bronchospasm in guinea-pigs induced by histamine, methacholine or leukotriene D (Raeburn *et al.*, 1994). In the same study it was shown that rolipram and RP73401 inhibited antigen-induced bronchospasm in sensitized animals and these bronchodilator effects were interpreted as a functional antagonism of airway contraction brought about by elevation of smooth muscle intracellular cyclic AMP.

In the present paper, the inhibition of CDP840 of antigen-induced bronchospasm is unlikely to be due to functional antagonism since higher doses of CDP840 did not modify the bronchoconstrictor response to histamine *in vivo*. Also it has been shown that CDP840 is a relatively weak inhibitor of histamine or methacholine-induced contraction of guinea-pig isolated tracheal rings (Holbrook *et al.*, 1996). The most likely explanation for the effects of CDP840 is through elevation of cyclic AMP in resident and recruited cells leading to inhibition of synthesis or release of bronchoconstrictor mediators such as histamine and the peptido-leukotrienes. Rolipram has been shown to inhibit the release of histamine from basophils and leukotrienes from isolated airways (Columbo *et al.*, 1993). Thus, rolipram does not inhibit leukotriene-induced bronchoconstriction *in vivo* but does prevent antigen-induced, leukotriene-dependent bronchoconstriction (Howell *et al.*, 1993). RP73401, however, has been shown to inhibit LTD_4 induced contraction (Raeburn *et al.*, 1994).

CDP840 did not augment the bronchodilator effects of salbutamol, differing from RP73401 and at variance with the hypothesis that β_2-adrenoceptors may be functionally linked to the PDE 4 isozyme (Tomkinson *et al.*, 1993). It is possible that an augmentation is only seen with PDE 4 inhibitors that possess intrinsic bronchodilator activity such as RP73401.

Another possible site of action for PDE 4 inhibitors in modulating airway responses is the sensory neurones. Rolipram but not inhibitors of PDE 3 or PDE 5 inhibits the excitatory noncholinergic neurotransmission in guinea-pig bronchi (Qian *et al.*, 1994; Undem *et al.*, 1994). Also, rolipram augments the nonadrenergic noncholinergic (NANC) relaxation of human isolated bronchus (Fernandes *et al.*, 1994). These effects could be related to the inhibition of the release of bronchoconstrictor neuropeptides such as substance P from the sensory neurones. This may also contribute to the anti-inflammatory effects of PDE 4 inhibitors since substance P is chemotactic for eosinophils (Smith *et al.*, 1993). Similarly, the inhibition of sensory neuropeptides may explain why rolipram is a potent inhibitor of the ozone-induced, neuronally-mediated bronchial hyperresponsiveness in guinea-pigs (Holbrook & Hughes, 1992). CDP840 is approximately 10 times more active than rolipram in suppressing hyperresponsiveness and RP73401 is relatively inactive (Holbrook *et al.*, 1996).

In summary, these studies demonstrate that CDP840, a potent, selective and novel inhibitor of PDE 4A, is a powerful inhibitor of allergen-induced bronchospasm and of inflammatory cell influx, specifically eosinophils. Its ability to reduce eosinophil infiltration and prevent their activation suggests that CDP840 should reduce the cytotoxicity, bronchoconstriction and hyperreactivity associated with eosinophil derived mediators (Gleich, 1990). Its specificity for eosinophils suggests that normal host defence mechanisms performed by other leukocytes such as neutrophils would be only marginally affected. Although CDP840 is a potent and selective PDE 4 inhibitor its pharmacological profile differs from that of rolipram and the more recently described PDE 4 inhibitors CP-80633 (Cheng *et al.*, 1995), RP73401 (Raeburn *et al.*, 1994), WAY PDA-641 (Howell *et al.*, 1994) and SB70449 (Underwood *et al.*, 1994). Unlike CP-80633 and rolipram, the activity of CDP840 is not modulated by endogenous catecholamines. CDP840 does not exhibit the potent bronchodilator activity of RP73401 and WAY PDA-641 but is significantly more potent in inhibiting antigen-induced bronchoconstriction and cell influx than SB70449. Furthermore, CDP840 exerts a marked suppression of ozone- and antigen-induced bronchial hyperresponsiveness. CDP840 is currently being evaluated in human asthma.

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(Received December 19, 1995
Revised March 13, 1996
Accepted March 21, 1996)