PDE 4 inhibitors: the use of molecular cloning in the design and development of novel drugs

Bernadette Hughes, Ray Owens, Martin Perry, Graham Warrellow and Rodger Allen

Phosphodiesterase 4 (PDE 4) enzymes are the principal phosphodiesterases responsible for the hydrolysis of cAMP in pro-inflammatory leukocytes. The functional consequences of elevating cAMP in these cells suggests that inhibition of PDE 4 offers a novel approach to asthma therapy. However, clinical development of early inhibitors has been limited by the side-effect of nausea. In this review, we detail how the molecular biology of the PDE 4 gene family has been integrated with biochemical, cellular and pharmacological studies. This approach has led to the discovery and development of CDP840, a prototype inhibitor for which efficacy has been demonstrated in a clinical model of asthma in the absence of side-effects.

In 1962, Butcher and Sutherland identified phosphodiesterases as the enzymes responsible for the breakdown of adenosine 3′,5′-monophosphate (cAMP)[1]. The discovery of the physiological importance of intracellular cAMP in mediating the effects of numerous hormones and the development of the concept of the 'second messenger' (Figure 1) were rewarded in 1971 with a Nobel Prize to Sutherland and his coworkers.

Studies performed over the past three decades have enabled the role of phosphodiesterases in a wide variety of physiological processes to be established. The powerful pharmacological properties of members of the methylxanthine family, such as caffeine and theophylline, which are partly the result of inhibition of phosphodiesterases[2], have made this group of enzymes an attractive target for the pharmaceutical industry. The synthesis of selective inhibitors and the application of molecular biology techniques have led to the classification of phosphodiesterases into seven different families (PDE 1–7; Table 1).

The type 4 enzyme (PDE 4), first identified in 1985 (Ref. 3), is specific for cAMP and is characterized by selective inhibition by rolipram, a compound synthesized in 1976 (Ref. 4) (Figure 2). Early studies on PDE 4 were directed to CNS indications such as depression, for which clinical trials using rolipram[5] and denbufylline (Figure 2) were conducted in the late 1980s (Refs 6,7). These studies proved inconclusive because side-effects such as nausea were observed before a statistically significant therapeutic effect had been established. The structural diversity of PDE 4 inhibitors, even of compounds such as rolipram and BRL61063 (Figure 2), that have exhibited such side-effects suggests that the effects can be attributed to inhibition of PDE 4 and not of an alternative target. More recent investigations have established that PDE 4 is the principal phosphodiesterase in inflammatory leukocytes, and thus the functional consequences of elevating cAMP in both airway smooth muscle and inflammatory leukocytes suggested that PDE 4 inhibition was a potential

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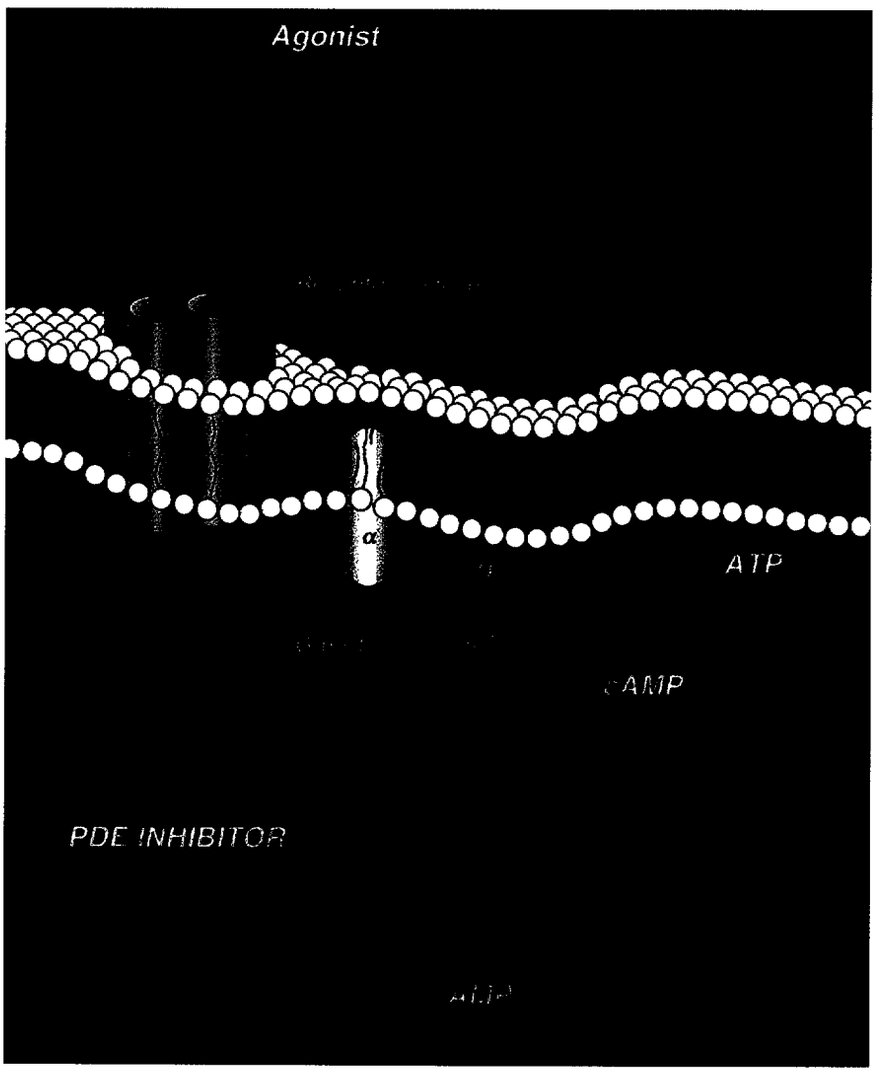

Figure 1. *Role of cAMP as the 'second messenger' in transducing receptor stimulation to intracellular changes. The site of action of phosphodiesterase (PDE) inhibition in this process is also shown.*

novel therapy in asthma[8]. In fact, theophylline (Figure 2), a nonselective phosphodiesterase inhibitor, has been the mainstay of asthma therapy for over three decades, even though it is associated with cardiovascular and gastric side-effects[9]. This suggests that selective PDE 4 inhibitors should be therapeutically effective agents in asthma. The challenge, therefore, was the discovery and development of novel PDE 4 inhibitors that retained the anti-inflammatory effects but had reduced undesirable side-effects.

The recent application of molecular cloning has revealed an unexpected complexity and diversity of PDE 4 enzymes. It is now known that there are at least four *PDE 4* genes, encoding distinct isoforms (A, B, C, D) that are differentially expressed in cells and tissues (Table 2). The availability of these enzymes as pure recombinant proteins in significant amounts offers the opportunity of exploiting their molecular diversity and varied cellular distribution to identify PDE 4 inhibitors with a superior therapeutic index.

Accordingly, in this review we describe how molecular biology has been integrated into biochemical, cellular and pharmacological studies in

Table 1. Cyclic nucleotide phosphodiesterase (PDE) gene families

Nomenclature	Gene family	Number of gene products	Number of splice variants	Selective inhibitors
PDE 1	Ca^{2+}/calmodulin-dependent	3	>9	Vinpocetine
PDE 2	cGMP-stimulated	1	2	MEP-I
PDE 3	cGMP-inhibited	2	>2	SKF94120
PDE 4	cAMP-specific	4	>15	Rolipram
PDE 5	cGMP-specific	2	2	Zaprinast
PDE 6	Photoreceptor	3	2	Zaprinast
PDE 7	cAMP-specific, rolipram-insensitive	1	2	?

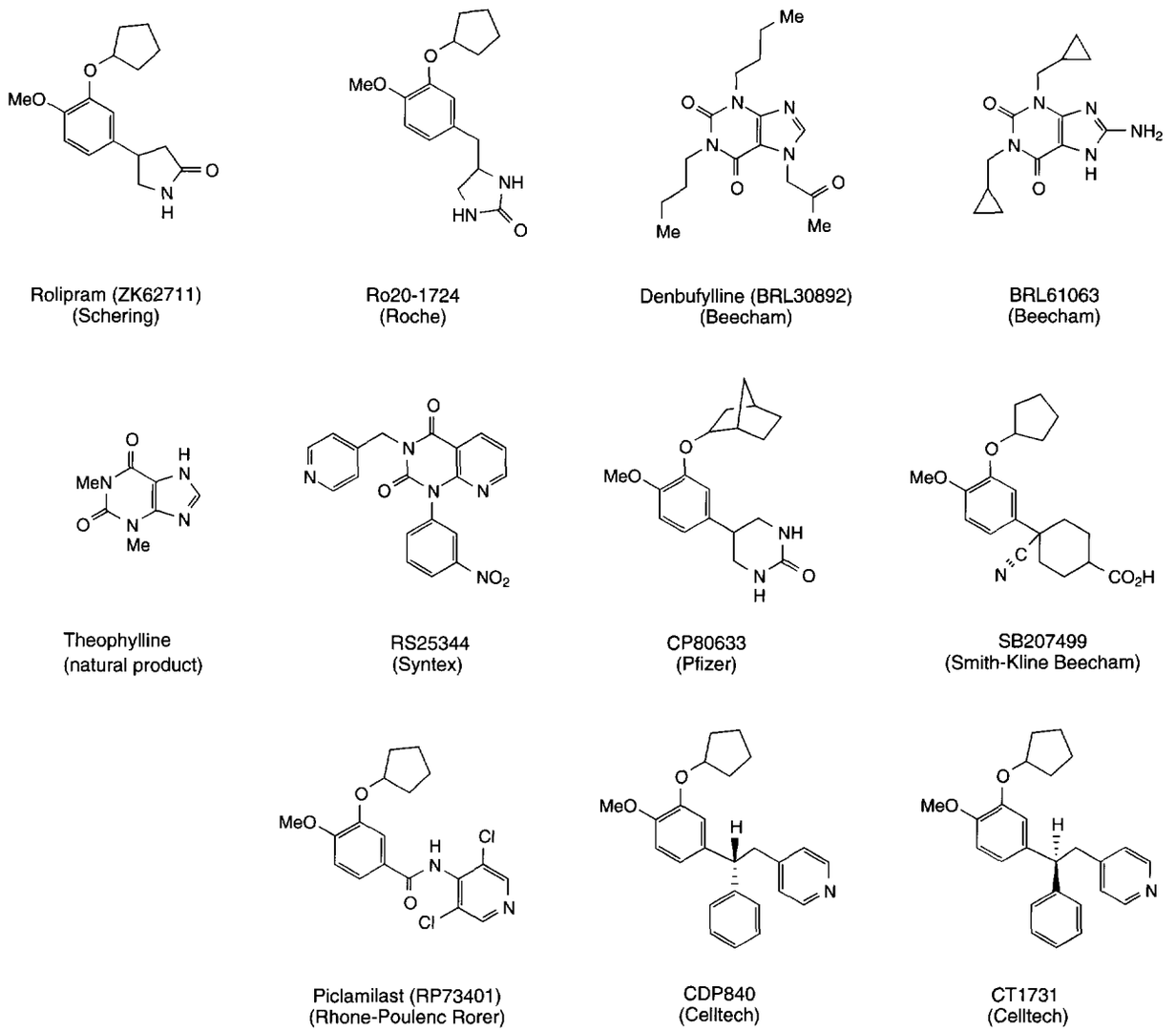

Figure 2. *Structures of the PDE 4 inhibitors described in the text. Some of these compounds have been clinically evaluated in both CNS and anti-inflammatory disorders such as asthma and atopic dermatitis. RP73401 is currently undergoing evaluation as an anti-arthritic agent.*

the development of orally active phosphodiesterase inhibitors for the treatment of asthma.

Molecular cloning and expression of PDE 4 enzymes

The first genetic handle on a cAMP phosphodiesterase came from studies of mutant fruit flies (*Drosophila melanogaster*) that were defective in olfactory learning. Normal fruit flies learn to avoid an odorant associated with an electric shock. An X-linked mutant, dunce (*dnc*), was isolated which failed to display this conditioned behaviour[10]. Subsequently, it was shown that the *dnc* mutant flies lack one of two cAMP phosphodiesterase activities that have been identified in *Drosophila*, resulting in an elevated cAMP content in these flies[11]. Davis and coworkers proposed that the *dnc* gene product was a phosphodiesterase[12]. This was confirmed by cDNA cloning and expression of the recombinant enzyme in yeast[13,14]. In addition, it has been shown that the single *dnc* gene gives rise to multiple RNA transcripts by a combination of alternative start sites and alternative RNA splicing. Thus a number of different enzyme variants may be obtained. This picture of enzyme diversity also holds true for mammalian type 4 phosphodiesterases.

Table 2. Distribution of PDE 4 mRNAs in human tissues[a]

Tissue	PDE 4 isoforms			
	A	B	C	D
Heart	++	++	ND	+
Brain	+++	+++	++	+++
Placenta	+	+	ND	+
Lung	++	++	ND	+++
Liver	+	+	ND	+
Skeletal muscle	+++	+++	+++	+++
Kidney	++	++	ND	++
Pancreas	++	+	ND	+
Spleen	+	++	ND	+
Thymus	+	+	ND	++
Prostate	+	++	ND	+++
Testes	++	+	+	+
Ovary	+	+	+	+
Small intestine	+	+	+	++
Colon	+	+	ND	++
PBL[b]	++	+++	ND	+++

[a]Northern blot analysis was carried out in a range of human tissues using *PDE 4* gene-specific probes. The abundance of each isoform mRNA (A–D) was scored as follows: +++ = strong expression, ++ = expression, + = weak expression; ND = not detected.
[b]PBL, peripheral blood leukocytes.

The molecular cloning of mammalian PDE 4 has revealed that there are four isoforms (A, B, C, D), each coded for by a separate gene, in rodents[15] and man[16]. Alignment of the full-length sequences of rat and human PDE 4 (Refs 17,18) has shown three regions of homology, namely two short upstream conserved regions (UCR1 and UCR2) and a central conserved region associated with catalytic activity[19]. There is greater than 90% sequence homology in the catalytic region between the rat and the human enzymes. Remarkably, this degree of conservation also extends to a comparison of the *dnc* and mammalian enzymes. Outside of the conserved regions, the isoforms are characterized by sequences specific for a particular subtype, which are conserved between different species. This implies some conservation of function for the four gene products.

A further level of enzyme diversity appears to be generated within each isoform. Thus, for each subtype, there are at least two variants that differ in their amino-terminal sequences. These may arise either from alternative initiation sites and/or from alternative splicing among the 5′-end exons. For human PDE 4B (Ref. 18) and PDE 4D (Ref. 19), long and short versions of the enzymes have been identified. The long-form enzymes comprise both UCR1 and UCR2 in addition to the catalytic region, whereas the short-form enzymes contain only UCR2, upstream of the catalytic region. The known isoforms of human PDE 4 are illustrated in Figure 3.

The functional significance of the different PDE 4 enzymes remains to be established. However, it is clear that expression of the different isoforms is regulated. Consequently, they display cell- and tissue-specific patterns of expression[20] (Table 2). For example, analysis of mRNA using RT-PCR indicates that isoform D may be the predominant PDE 4 in guinea-pig eosinophils[21].

The molecular diversity of PDE 4 clearly offers opportunities for specifically targeting a particular isoform for inhibition and hence to achieve a particular functional effect. However, the low abundance of the native enzymes from natural sources and their susceptibility to proteolysis has limited the purification of intact enzymes for biochemical characterization and inhibitor screening.

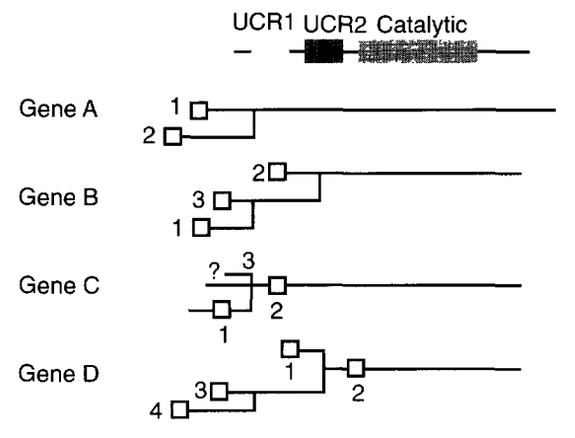

Figure 3. *Schematic representation of human PDE 4 mRNAs. The different mRNAs generated from each of the four human* PDE 4 *genes (A–D) are shown in solid lines aligned to the domain structure of the protein, shown by the filled boxes. Regions of variant sequence in the mRNAs are indicated by the branched lines and numbered. The position of the putative translation start sites of each mRNA is shown by an open box.*

Table 3. Kinetics of cAMP hydrolysis by human recombinant PDE 4 isoenzymes

Gene	Host[a]	K_m (μM)	V_{max} (μmol/min/mg)[b]
A	Yeast	3.1	4.6
	COS	7.6	–
	SF9	4.3	3.8
B	Yeast	4.1	6.0
	COS	4.3	–
	SF9	–	–
C	Yeast	2.5	0.6
	COS	0.6	–
	SF9	1.1	0.3
D	Yeast	1.8	11
	COS	2.1	9
	SF9	–	–

[a]COS, a monkey epithelial cell line; SF9, *Spodiptera frugiperda* cells.
[b]Hill coefficient ≈1.0 in all cases.

A further difficulty is the likely coexpression of one or more isoforms in a given cell or tissue source. Thus, attention has focused on the expression of cDNAs to produce recombinant enzymes of defined isoform. PDE 4 cDNAs have been expressed using a number of host cells, including mammalian, yeast and insect cells[18,22,23].

The isolation of full-length PDE 4 cDNAs has proved technically challenging because of the low abundance of the mRNAs (approx. 1 part in 10^6 of the mRNA fraction) and the length of the transcripts (up to 7 kb). We therefore evaluated a variety of sources to obtain representative full-length PDE 4 cDNAs. Thus, human PDE 4A cDNA was obtained from a frontal cortex cDNA library, while cDNAs for PDE 4B and PDE 4D were isolated from a human eosinophil cDNA library. PDE 4C, which demonstrated a much more restricted tissue distribution, was isolated from an astrocytoma cell line. With the exception of PDE 4B, all cDNAs corresponded to long-form splice variants containing both UCR1 and UCR2 domains. The PDE 4B was the short-form splice variant that lacks UCR1. These cDNAs were inserted into a vector under the control of the human cytomegalovirus major immediate early gene promoter for expression in mammalian cells (e.g. COS cells, a monkey epithelial cell line).

In addition, inducible expression in yeast (*Saccharomyces cerevisiae*) was obtained by inserting the PDE 4 cDNAs into a yeast vector with a high copy number under the control of the galactose promoter. Recombinant baculoviruses were constructed by cotransfecting appropriate transfer vectors with linearized DNA from AcNPV (*Autographa californica* nuclear polyhedrosis virus). Expression of recombinant enzymes was carried out by infection of *Spodiptera frugiperda* (SF9) cells.

Purification of recombinant PDE 4 enzymes

Biochemical quantities of recombinant PDE 4 enzymes were produced by expression in yeast and insect cells. In general, higher yields of product were obtained using the baculovirus system. All enzymes were purified from soluble cell lysates using a combination of anion-exchange (Q Sepharose), dye (Blue Sepharose) and affinity (AAL115) chromatography[24]. This yielded highly active enzymes (V_{max} = 1–11 μmol cAMP/min/mg) with all isoforms exhibiting a similar K_m for cAMP (0.6–7.6 μM) (Table 3). Although the recombinant enzymes exhibited aberrant higher molecular weights on SDS-PAGE, this was consistent with observations with native PDE 4 isoenzymes isolated from tissue or cell sources (Table 4). All enzymes exhibited Michaelis–Menten kinetics with respect to cAMP. Linear double-reciprocal plots over a broad range of substrate concentrations gave no indication of cooperativity.

PDE 4 inhibitor interactions: Sc/Sr

The availability of significant quantities of purified PDE 4 enzymes has enabled detailed studies to be performed on the mechanism of inhibition by a selection of structurally diverse compounds (Figure 2).

Table 4. Molecular weight (g/mol) of PDE 4 isoenzymes by SDS-PAGE and western blotting

	Phosphodiesterase isoform			
	A	B	C	D
Expected	98,118	64,347	67,714	76,329
Recombinant[a]	117,000	78,000	81,000	98,000
Brain	117,000	78,000	–	98,000
U937	117,000	–	–	98,000
U87	117,000	78,000	81,000	98,000
HL60	–	78,000	–	98,000
Jurkat J6	117,000	–	–	98,000

[a]Expressed in yeast, COS cells and SF9.

Table 5. Inhibition (IC_{50}, nM) of human recombinant PDE 4 isoenzymes expressed in COS cells

	PDE 4A	PDE 4B	PDE 4C	PDE 4D
RS25344	11	1.9	0.25	0.1
RS14203	1.1	0.4	3.3	0.25
(*R*)-Rolipram	103	182	23	219
(*S*)-Rolipram	330	311	342	311
Denbufylline	123	73.5	90.7	29.3
SB207499	50.1	40.5	134	9.6
RP73401	0.5	0.5	1.0	0.1
CDP840	3	4.3	106	2.9

The potency of known PDE 4 selective inhibitors against the four isoenzymes is given in Table 5. PDE 4C was usually less sensitive to all inhibitors, although, interestingly, certain inhibitors such as (*R*)-rolipram and RS25344 (Figure 2) were more potent. With respect to isoenzymes A, B and D, there was no evidence of inhibitor isoenzyme selectivity (as defined by a 10-fold difference in IC_{50}), except that most inhibitors, in particular RS25344, were more potent against PDE 4D. This was of particular interest given the recent findings of Conti and coworkers[18,25] that PDE 4D can be phosphorylated by cAMP-dependent protein kinase A and that the phosphorylated enzyme is much more sensitive to RS25344 and related structures. This concept of conformational change leading to differential inhibitor sensitivity will be addressed later in this review.

The log dose–inhibition curves indicated that certain inhibitors such as RS25344, (*R*)-rolipram and denbufylline gave shallow profiles (Hill coefficient <1), whereas others such as SB207499, RP73401 (Figure 2) and (*S*)-rolipram gave response curves with Hill coefficients close to unity. The former compounds also did not appear to be simple competitive inhibitors because the change in slope of double-reciprocal plots of inhibition was lower than expected, and was concave in form. This would be expected if these inhibitors were interacting with the enzyme with at least two distinct affinities.

Analysis of the Dixon plots for (*R*)-rolipram and RS25344, which were nonlinear, gave two distinct K_is for each compound, which, in the case of RS25344, were three orders of magnitude apart (Table 6). Dixon plots for compounds such as SB207499 were linear and gave a single K_i in close agreement with the IC_{50}. This ability of certain inhibitors to display differing affinities for PDE 4 supports the findings of

Table 6. Inhibitor K_i determined from Dixon plots using human recombinant PDE 4A

	K_i^1 (nM)	K_i^2 (nM)
(*R*)-Rolipram	28	237
RS25344	0.9	2230
SB207499	53	–

Souness and coworkers[26], who observed that the IC_{50} values for rolipram and denbufylline against an eosinophil PDE 4 could be shifted 10-fold by treatment of the enzyme with sodium deoxycholate or sodium vanadate–glutathione. A similar shift in IC_{50} was not observed for other inhibitors such as isobutylmethylxanthine.

The ability of rolipram to interact with a high-affinity binding site had been suggested initially from binding studies with [^{3}H]rolipram and brain membranes[27]. The affinity of ≈2 nM was in good agreement with the plasma levels required for efficacy in models of depression. The CNS effects of rolipram correlated much better with high-affinity binding than with the IC_{50} for inhibition of catalytic activity of the isolated enzyme. Subsequently, Torphy and coworkers[22,28] demonstrated that this high-affinity rolipram binding site (Sr) existed in a truncated recombinant PDE 4A and a full-length recombinant PDE 4B.

We have identified Sr on all four isoenzymes (Table 7) with K_d values between 1 and 5 nM, although, interestingly, PDE 4C expressed in yeast failed to exhibit an Sr. However, examination of the stoichiometry of binding indicated that only 1–10% of the total enzyme expressed a high-affinity binding site for [^{3}H]rolipram (Table 7). This suggested that these enzyme preparations were conformationally heterogeneous. In order to explore this further, we produced a truncated version of PDE 4A, lacking the first 300 amino acids,

Table 7. [^{3}H]Rolipram binding by human recombinant PDE 4 isoenzymes[a]

Gene	Source	K_d (nM)	Stoichiometry
A	Yeast	3.7	0.005–0.04
B	Yeast	2.4	0.009–0.05
C	Yeast	ND[b]	–
	SF9	2.6	0.02
D	Yeast	1.8	0.01–0.08

[a]In all cases: Hill coefficient ≈1.0; linear Scatchard; inhibited by cAMP.
[b]ND, not detected.

Table 8. Inhibition of human recombinant PDE 4A and human recombinant PDE 4A330

	IC_{50} (nM)	
	PDE 4A	PDE 4A330
RS25344	11	753
RS14203	1.1	39
(*R*)-Rolipram	103	501
Denbufylline	123	537
SB207499	50.1	45.3
RP73401	0.5	0.4
CDP840	6.9	3.9

which, as a consequence, did not express Sr but exhibited identical substrate kinetics to those of the full-length enzyme. Interestingly, compounds such as SB207499, RP73401 and CDP840 (Figure 2) were equally active against both forms of the enzyme, whereas (*R*)-rolipram, denbufylline and RS25344 were significantly less active against the truncated enzyme (Table 8). Thus it would appear that the presence of Sr has a profound effect on the ability of certain inhibitors such as (*R*)-rolipram and RS25344 to inhibit the catalytic activity of PDE 4 isoenzymes.

The availability of PDE 4 cDNA also offers the opportunity of probing the structure–function relationship of the enzymes by site-specific mutagenesis. Of particular interest to the development of selective inhibitors is an understanding of the residues involved in inhibitor binding. Pillai and coworkers[29] recently identified a single amino acid substitution (alanine for aspartate) in the catalytic region of PDE 4B, which is critical for inhibition by rolipram, using random mutagenesis of the rat cDNA expressed in yeast. We have introduced this change into human PDE 4A by site-directed mutagenesis and shown a similar loss of rolipram inhibition. This appears to be a class effect because the related inhibitors, RP73401 and Ro201724 (Figure 2), also lose potency on the mutant enzyme.

Effects of elevating intracellular cAMP

There is an extensive literature describing the effects of phosphodiesterase inhibitors on various inflammatory cell responses such as elevation of cAMP and inhibition of superoxide production, degranulation, chemotaxis and tumour necrosis factor (TNF) release in eosinophils, neutrophils and monocytes[21,30–35]. We have utilized some of these assays as screens for profiling and selecting inhibitors to go forward into various pharmacological models. In addition, the performance of a range of inhibitors in some of these assays has been used to investigate the relationship between elevation of cAMP and inhibition of cell function, as well as to elucidate the role of the catalytic site (Sc) and the high-affinity rolipram binding site (Sr) in achieving these effects in the cell.

We have investigated the ability of two enantiomeric pairs, (*R*)- and (*S*)-rolipram and CDP840 and its less active enantiomer CT1731, to elevate cAMP in a range of cell types; the results are shown in Table 9. The elevation of cAMP in each of the four cell types, despite different stimulation conditions, demonstrates a stereoselective effect when the two enantiomeric pairs are compared. The selectivity ratio for the eutomer over the distomer is greater in the guinea-pig eosinophil and the primary bronchial smooth muscle cell line (10–20-fold) than in the human neutrophil and the U87 neuronal cell line (3–5-fold). This may be related to the different stimulation conditions utilized for the various cell types. The U87 cells were treated with the phosphodiesterase inhibitor alone while the human neutrophils were stimulated with dihydrocytochalasin B (dhCB) and *N*-formyl-Met-Leu-Phe (fMLP). However, the guinea-pig eosinophils and the bronchial smooth muscle cells were

Table 9. The effect of phosphodiesterase inhibitors on elevation of cAMP in various cell types[a]

	(*R*)-Rolipram	(*S*)-Rolipram	CDP840	CT1731
Human neutrophils[b]	1.84	0.57	0.53	0.1
Guinea-pig eosinophils[b]	1.12	0.12	0.564	0.042
U87[c]	921	3725	282	1067
Human bronchial smooth muscle cells[d]	39.8	760	235	NT

[a]The data presented are the relative potencies of two enantiomeric pairs of phosphodiesterase inhibitors in elevating cAMP in various cell types.
[b]Potency ratios relative to rolipram.
[c]EC_{50} values (nM).
[d]Expressed as the concentration of inhibitor required to double the baseline cAMP concentration.

stimulated with isoprenaline, a β_2-adrenoceptor agonist, which is coupled to adenylyl cyclase. This activation of the cyclase may accentuate stereoselective differences between the enantiomers.

The other interesting observation that emerges from the data in Table 9 is that (*R*)-rolipram is a more potent elevator of cAMP than CDP840 in human neutrophils, guinea-pig eosinophils and human bronchial smooth muscle cells (2–6-fold), whereas in the U87 neuronal cell line CDP840 is three times more potent than (*R*)-rolipram in elevating cAMP. CDP840 is also a more potent elevator of cAMP than rolipram in another neuronal cell line, NG115. The explanation for this switch in rank order of potency may be a consequence of PDE 4 isozyme differences or perhaps conformational differences of the same isozyme.

We investigated whether using different stimuli to elevate cAMP in guinea-pig eosinophils would affect the potency determined for a range of PDE 4 inhibitors. No significant differences were observed in rank order of potency, or absolute values, when prostaglandin E_2 (PGE_2) was used to stimulate the eosinophils compared with isoprenaline.

Elevation of cAMP leads to activation of protein kinase A and subsequent inhibition of signalling and cell response as a result of a critical phosphorylation(s). We investigated the relationship between elevation of cAMP and inhibition of superoxide production in dhCB- and fMLP-stimulated human neutrophils treated with rolipram. The results are shown in Figure 4. The inhibition of superoxide production was determined over 4 minutes; then the cells were immediately harvested and prepared for cAMP determination. There is a good correlation between elevation of cAMP and inhibition of superoxide production. As soon as elevation of cAMP above baseline can be detected, inhibition of the cell response is observed. Maximum inhibition of superoxide is observed with a modest elevation of cAMP, although the cAMP can be elevated significantly higher with no further effect on the cell response. This effect can also be observed for various PDE 4 inhibitors with a wide range of potencies and cAMP-elevating capabilities. Initial increases in the level of cAMP over baseline give corresponding increases in inhibition of superoxide production up to a plateau, over and above which even a large cAMP elevation does not translate into further inhibition of the cell response.

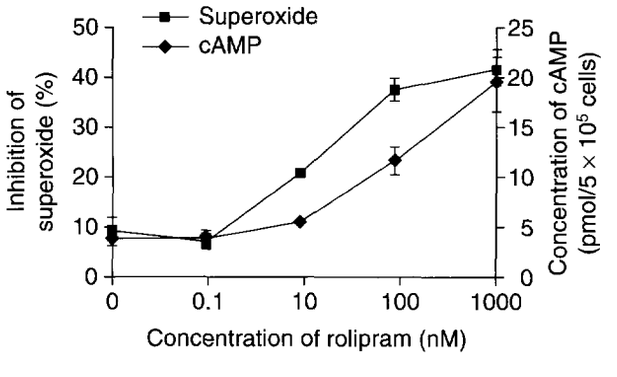

Figure 4. *The effect of rolipram on superoxide anion generation and cAMP in* N*-formyl-Met-Leu-Phe stimulated human neutrophils* (n = *11, mean ± SEM).*

The role of binding to the Sc or the Sr, in terms of elevation of cAMP and inhibition of cell responses, is not fully understood. However, we have a range of compounds that have different affinities at the Sc and the Sr, and these are tools to help understand the consequences of binding with high affinity at one or other site or both. Figure 5 shows the relationship between elevation of cAMP and inhibition of superoxide production when compared with binding at the Sc or the Sr. It is clear that elevation of cAMP and inhibition of superoxide production correlate equally well with binding at the Sc and the Sr for this selection of phosphodiesterase inhibitors. These findings are in contrast to the recently published data of Barnette and coworkers[30], who found that inhibition of superoxide production in guinea-pig eosinophils was more closely correlated with binding at the Sc than with binding at the Sr. This same group subsequently reported[36] the finding that increased acid secretion in isolated gastric glands from the rabbit showed a correlation in rank order to the rank order of binding at the Sr, but not at the Sc. However, the inhibitors tested in the two systems were not identical, and, moreover, they are different to the inhibitors used in the present study.

Therapeutic effects of PDE inhibitors

Asthma is a chronic inflammatory disease characterized by airway constriction, hyper-responsiveness and ongoing cellular inflammation. These effects are a consequence of inflammatory mediators such as histamine and leukotrienes acting together with cytokines such as IL-5 from activated T cells. The ability to inhibit inflammatory mediator release, modulate immune cell function and thus prevent allergen-induced bronchoconstriction and the ensuing pulmonary cell influx are essential activities of a compound for it to have therapeutic efficacy in asthma. Thus it is important to determine whether an interaction with the PDE 4 target

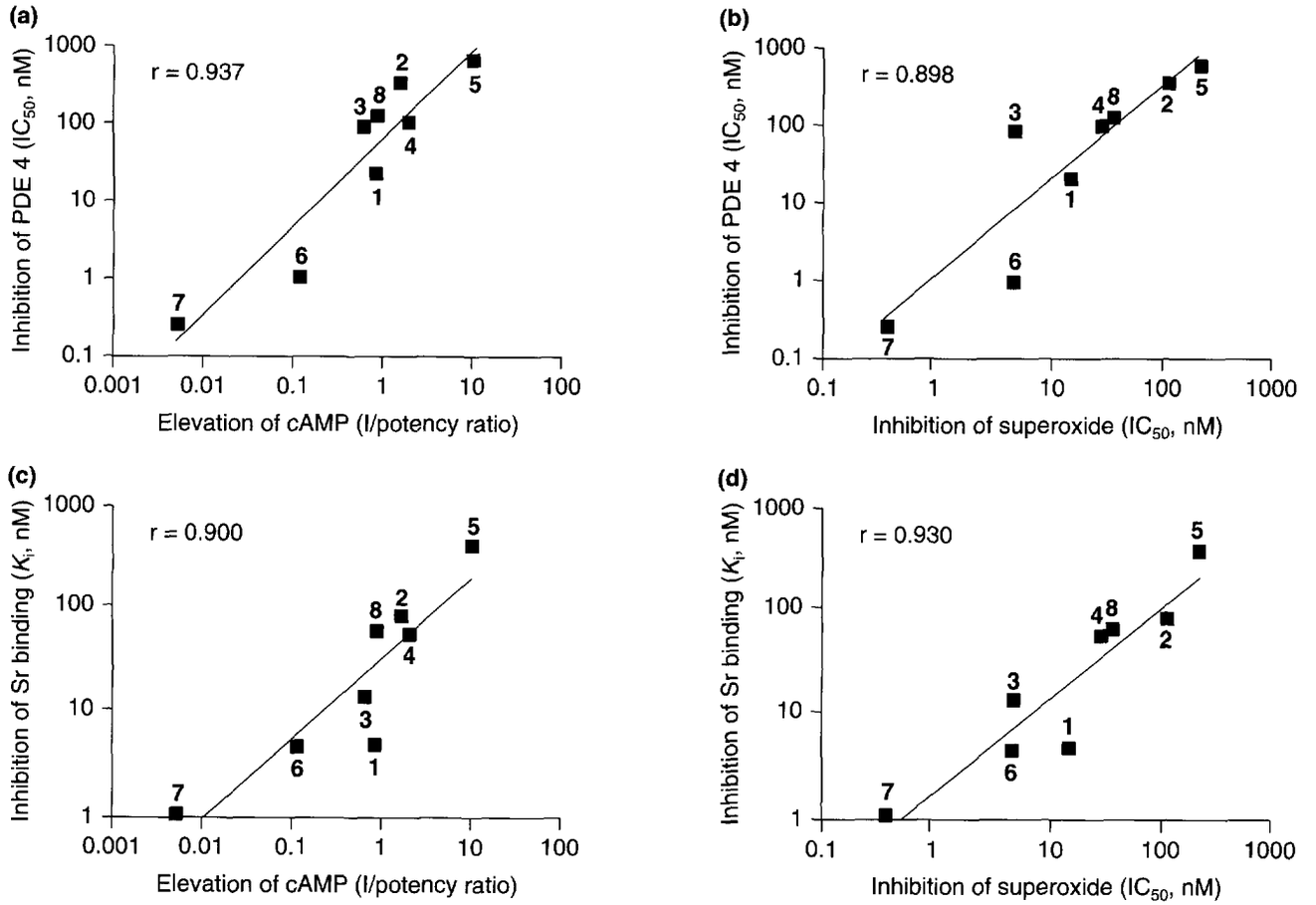

Figure 5. *The correlation of cAMP elevation (a, c) and inhibition of superoxide anion production (b, d) in* N*-formyl-Met-Leu-Phe stimulated human neutrophils with the inhibition of PDE 4 catalytic activity (a, b) and binding at the rolipram binding site (Sr)* (c, d)*. Key to compounds:* ***1****, (R)-rolipram;* ***2****, (S)-rolipram;* ***3****, denbufylline;* ***4****, CDP840;* ***5****, CT1731;* ***6****, RP73401;* ***7****, RS25344;* ***8****, SB207499 (see Figure 2 for structures).*

enzyme at the Sc, Sr or indeed both is necessary to bestow this profile on an inhibitor. It is possible to model the various aspects of the disease in experimental animals and we have used these well-characterized animal models to evaluate the efficacy of the PDE 4 inhibitors. The importance of the Sr in influencing the pharmacological profile of PDE 4 inhibitors has not fully been elucidated and it may have important implications in predicting not only efficacy but also the side-effects of nausea and headache[5], which have hampered the clinical development of PDE 4 inhibitors to date.

The stereoisomers of rolipram display only a 2–3-fold difference in potency in inhibiting the Sc but an approx. 20-fold difference in inhibiting the Sr (Table 10). In contrast, CDP840 and CT1731 display an approx. 16-fold difference in catalytic activity and an approx. 7-fold difference at the Sr. The availability of these agents together with others, such as RP73401 (Ref. 37), RS25344 and SB207499, which exhibit different activities at the two sites, provides tools that have allowed us to probe their importance in predicting pharmacological efficacy and the side-effect profile.

Direct effects on bronchial smooth muscle

Elevation of cAMP will mediate relaxation in airway smooth muscle; indeed, the second messenger system of the β_2-adrenoceptor is cAMP, and it has been hypothesized that the β_2-adrenoceptor is linked to PDE 4 (Ref. 38). Generally, PDE 4 inhibitors are weak bronchodilators, exhibiting significantly less efficacy than the β_2-agonist salbutamol (Figure 6). They are also less effective than the mixed PDE 3/4 inhibitors[38,39]. However, some PDE 4 inhibitors, such as

Table 10. Effects of phosphodiesterase 4 (PDE 4) inhibitors on inhibition of allergen-induced bronchoconstriction and reversal of histamine-induced contraction of guinea-pig trachea

PDE 4 inhibitor	Sc[a] (IC_{50}, nM)	Sr[b] d(K_i, nM)	AB[c] (IC_{50}, nM)	HT[d] (IC_{25}, nM)
RS25344	11	1	6	10
RP73401	1.1	5	2	2
(*R*)-Rolipram	103	5	300	7
(*S*)-Rolipram	330	91	600	45
CDP840	6.9	60	300	3,700
CT1731	151	415	3,500	1,600
Denbufylline	123	19	5,000	–
SB207499	50.1	77	300	

[a]IC_{50} versus PDE 4A.

[b]High-affinity rolipram binding from guinea-pig brain membranes.

[c]AB = inhibition of allergen-induced bronchoconstriction.

[d]HT = reversal of histamine-induced tone.

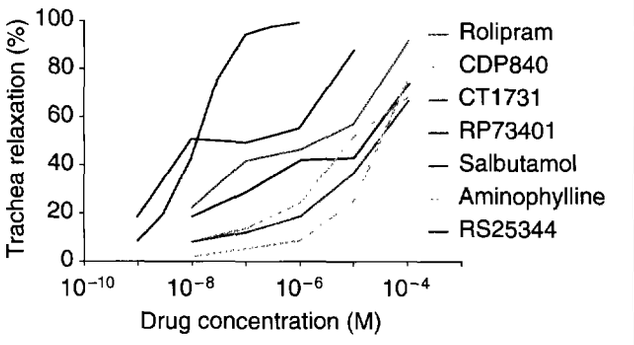

Figure 6. *The relaxant effects of PDE 4 inhibitors and salbutamol in guinea-pig trachea precontracted with histamine. Data are mean ± SEM (n = 6).*

rolipram, RP73401 and RS25344, are effective bronchodilators, exhibiting a biphasic curve in reversing histamine-contracted trachea (Figure 6), whereas others, such as CDP840 and SB20499 (data not shown), show a monophasic curve. The biphasic curve suggests multiple mechanisms of action, whereas that of CDP840 indicates a single mechanism. The relative potencies of these compounds for relaxing airway smooth muscle does not correlate with inhibition of PDE 4 at the Sc. Furthermore, there is no direct relationship with affinity at the Sr because (*R*)- and (*S*)-rolipram show only a 2–3-fold potency difference[40,41] and CDP840 and CT1731 also display little difference in activity. However, compounds such as rolipram, RS25344 and RP73401 are more potent bronchorelaxants and these do tend to have a higher affinity for the Sr. Early studies by Harris and coworkers[39], employing a large range of PDE 4 inhibitors, did find a correlation with binding to the Sr. These data suggest that activity at both sites is important, but that activity at the Sr confers greater potency for bronchodilator effects than at the Sc.

Inhibition of allergen-induced bronchoconstriction

The effects of a range of PDE 4 inhibitors on the inhibition of allergen-induced bronchoconstriction are shown in Table 10. The most potent compounds in this assay are again RS25344 and RP73401, but a bronchodilator effect will contribute to efficacy in this system. There is no doubt that the nanomolar potency of these two compounds results from the summation of a relaxant effect and inhibition of bronchoconstrictor mediators from sensitized tissue. There is only a 2-fold difference between (*R*)- and (*S*)-rolipram in inhibiting this response, and a 10-fold difference between CDP840 and CT1731, both potency ratios being very similar to the ratio at the Sc rather than at the Sr. Denbufylline exhibits a similar lack of effect despite having a K_i of 19 nM at the Sr, with considerably less activity at the Sc (IC_{50} = 123 nM). These data suggest that compounds with potency at the Sc exhibit superior efficacy in this assay than those, such as denbufylline, that have potency at the Sr. However, the most potent activity is seen with compounds that exhibit good and comparable activity at both sites, such as RP73401 and RS25344.

Inhibition of pulmonary cell influx and bronchial hyper-reactivity

Numerous studies have demonstrated the ability of PDE 4 inhibitors to reduce allergen-induced pulmonary cell influx. In a sensitized guinea-pig model[40,42], a neonatally sensitized rabbit model[43] and primate models naturally sensitive to *Ascaris suum*[44,45], both rolipram and CDP840 have inhibited both inflammatory cell influx and bronchial hyper-responsiveness. However, CDP840, in contrast to rolipram, also inhibits antigen-induced acute bronchoconstriction in both rabbit and primate models[43,44,46] and, in the rabbit, exhibits an anti-inflammatory profile comparable with that of the steroid budesonide[43]. Although direct correlation of *in vitro* potency with *in vivo* potency is complex because of the differential metabolic and pharmacokinetic profiles that different compounds may exhibit, the anti-inflammatory effects are more consistent with activity at the Sc rather than at the Sr. The differential activity of CDP840 and CT1731

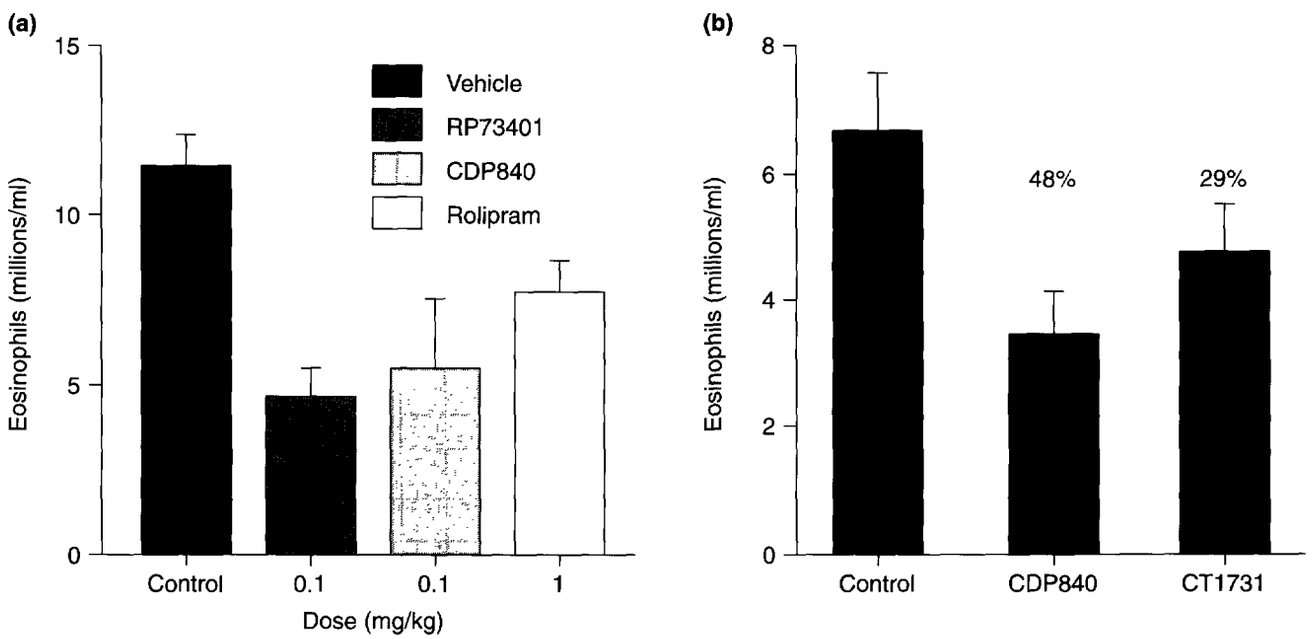

Figure 7. *(a) The effects of PDE 4 inhibitors on antigen-induced eosinophil recruitment into guinea-pig bronchoalveolar lavages. Each value is the mean ± SEM of 6–10 animals. (b) Stereoselective inhibition of eosinophilia in guinea-pig by CDP840 and CT1731* (n = 7–8, *mean ± SEM). Dose of CDP840 = 1 mg/kg, 3 times daily, intraperitoneal.* P *<0.05 (analysis of variance) compared with the control.*

is about 10–100-fold in inhibiting eosinophil influx in guinea-pigs (Figure 7a)[43], and greater than 40-fold in inhibiting ozone(neuronally)-induced hyper-responsiveness[47]. Rolipram is less active in inhibiting eosinophil infiltration (1 mg/kg, intraperitoneal), and RP73401, although equipotent with CDP840 in inhibiting eosinophilia (Figure 7b), was considerably less active in inhibiting hyper-responsiveness[47]. RS25344, which has a greater affinity for the Sr than for the Sc, also displays poor activity in a rat eosinophilia model, and SB207499, which has comparable activity at the Sr and the Sc but is less active than CDP840 at the Sc, requires doses of ≈30 mg/kg (oral) for anti-inflammatory activity[48].

Thus, a key activity for an anti-asthma compound, the anti-inflammatory action, appears to be more dependent on activity at the Sc than at the Sr. Similarly, the prevention of hyper-responsiveness, certainly of a neuronal stimulus, may also be determined by inhibitory activity at the Sc rather than at the Sr.

Relevance of adverse events to efficacy in man

The major adverse effects of PDE 4 inhibitors have been nausea and headache, which are the most commonly observed side-effects of this class of compound following administration to man (see Box 1). These side-effects occur both with and without efficacy. Consequently, there has been debate as to whether efficacy and adverse events are inextricably linked. There are a number of sites at which PDE 4 inhibitors may act to elicit these effects, including:

- a direct effect on gut motility/irritancy via their ability to moderate both inhibitory and excitatory nonadrenergic, noncholinergic neuronal function[41,49];
- augmentation of gastric acid release[36];
- elevation of cAMP in the area postrema, which has long been associated with induction of nausea and emesis in animal models[50];
- direct action on the vomiting centre.

It has been hypothesized that inhibitors that do not penetrate the CNS may be less emetic; however, this strategy has not been successful[51]. Similarly, administration by inhalation of PDE 3/4 inhibitors has not overcome the side-effect profile[52]. The importance of activity at the Sr versus activity at the Sc has obviously been a key question in determining the profile of adverse effects.

Box 1. Clinical experience with phosphodiesterase 4 inhibitors

Drug	Dose and effect	Reference
Rolipram	2.25 mg/day for 28 days (oral) • modest clinical benefit • adverse events: nausea	5
BRL61063	0.5–3.5 mg/day (oral) in a single dose • trend to 50% reduction in tumour necrosis factor-α production at 3.5 mg/day • adverse events: nausea, vomiting from 0.5 mg/day	55
CDP840	15 mg (oral) twice daily for 10 days • significant inhibition of late-phase response • adverse events: none	54

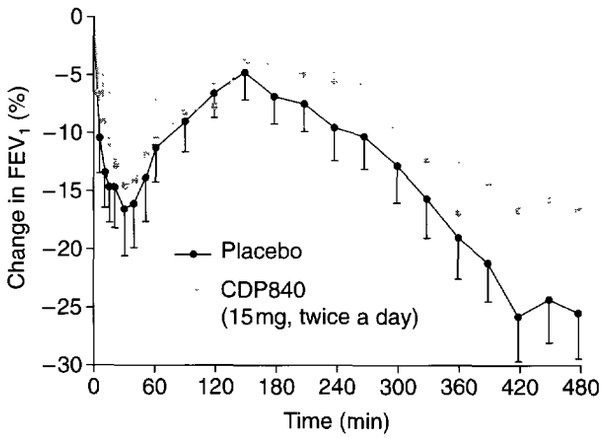

Figure 8. *Effect of 9.5 days of treatment with CDP840 15mg, twice daily, on the response to allergen, expressed as the percentage fall in mean FEV_1 ± SEM over the 8 h period following exposure (n = 13; FEV_1, forced expiratory volume in one second).*

Recently, it has been suggested that compounds that have a high affinity at the Sr can be correlated with emesis in dogs and also with increased gastric acid secretion[36]. Both RS23544 and RP73401 elicit emesis in experimental animal models at doses similar to that of rolipram and all have high affinity at the Sr (Table 10). Similarly, CP80633 (Figure 2) has a 10 nM affinity for the Sr and is also emetic *in vivo*[53]. CDP840 and SB207499 are PDE 4 inhibitors with a comparatively lower affinity at the Sr, but CDP840 differs significantly from SB207499 in having a significantly higher potency at the Sc. CDP840 has been administered to man in doses up to 30 mg twice daily without any adverse events of nausea or headache. In a clinical model of asthma, an allergen challenge study in asthmatics, CDP840 at 15 mg twice daily resulted in a significant inhibition of the late asthmatic response (P = 0.018) (Figure 8). These studies have shown that this is not the result of a direct bronchodilator effect or of antagonism of histamine, and the results indicate that inhibition of PDE 4 by CDP840 causes an anti-inflammatory effect in the lung[54] without side-effects. These data demonstrate that it is possible to separate efficacy from the adverse events of nausea and headache in man.

Summary

Inhibitors of the PDE 4 isozyme have been identified as novel anti-inflammatory agents for the treatment of asthma; however, clinical development of these agents has been hampered by the adverse effects of nausea and headache. We have applied a multidisciplinary approach to the development of novel PDE 4 inhibitors that lack dose-limiting side-effects. Molecular cloning has revealed that PDE 4 activity results from the products of four independent genes. These in turn give rise to a number of splice variants, which are differentially expressed in cells and tissues. The availability of the PDE 4 enzymes as pure recombinant proteins has enabled mechanistic studies to be performed, culminating in the synthesis of a novel series of PDE 4 inhibitors. From an *in vitro* and *in vivo* pharmacology screening cascade, a prototype inhibitor, CDP840, was identified from this series, which has shown efficacy in the absence of side-effects in a clinical model of asthma. The involvement of PDE 4 in other inflammatory, immune and depressive disorders illustrates the potential of this approach in the identification of further novel agents.

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